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SUMMARY

The effective evaluation of the neurotoxic potential of chemicals is reviewed in order to provide adequate safeguards for human health. The effect of a chemical on the nervous system is difficult to assess because of the complexity of the nervous system. Essentially all substances could be classified as "neurotoxic" since all chemicals are toxic at some dose level and will induce some behavioural change at these dose levels. Therefore, detection of those compounds that may cause direct, persistent, adverse effects on the nervous system should be given the most critical attention in order to protect human health in the workplace and the environment.

Evaluation of the neurotoxic potential of a chemical should include descriptions of functional and morphological effects as well as the determination of the dose response, time course, reversibility of effects and the no observed effect level (NOEL). Differentiation between direct and indirect neurotoxic effects of a substance is critical for scientific and regulatory considerations. Also, investigation of species specificity, toxicokinetics and the mechanism of a neurotoxic effect may provide useful information.

A tiered testing strategy is recommended for the evaluation of the neurotoxic potential of chemicals. This approach should be flexible and guided by factors such as the structure and physical form of the chemical, the potential human exposure and the nature of any neurotoxic effect.

Evaluations of the nervous system in the context of standard toxicity studies are appropriate screening tests (Tier 1) for the detection of potential neurotoxicity. Standard toxicity studies may be conducted at relatively high dose levels with different durations and routes of administration and examine several species of animal. There are numerous examples in the scientific literature that demonstrate the ability of standard toxicity studies to detect a variety of neurotoxic effects.

Validated methods for the evaluation of the nervous system should be included in standard toxicity studies. Some guidelines for standard toxicity studies should be strengthened by the inclusion of additional clinical and pathological examinations related to the nervous system. Evaluation of pups in standard reproductive studies could include physical and functional landmarks covering sensory and motor functions. Additional emphasis on the evaluation of the nervous system within the context of standard toxicity studies would eliminate the need for separate guidelines for neurotoxicity screening. Data relevant for identification of potential neurotoxicity from available standard toxicity studies could be summarised and evaluated in a separate report.

Studies specifically designed to assess neurotoxicity (Tier 2 studies) should be performed with chemicals for which there is an indication of a neurotoxic potential and the available data are inadequate for risk assessment. The goal of these tests is to evaluate thoroughly the nervous system with broad, exploratory tests that will provide adequate data for risk assessment. Tier 2 studies should evaluate the major functions and structures of the nervous system by comprehensive clinical examinations and neuropathological assessment. These studies may be conducted in conjunction with standard toxicity studies such as subchronic studies so that any potential neurotoxicity can be interpreted in the context of other systemic toxicity. Tier 2 tests will be adequate for risk assessment in most cases and only rarely would further testing be required to provide additional information essential for the protection of human health.

More specific neurotoxicity tests may be conducted for advanced characterisation of discovered neurotoxicants (Tier 3) with carefully selected techniques such as neurophysiology, biochemistry, observation of behaviour or neuropathology. Investigation of the potential of the chemical to induce developmental neurotoxicity also may be considered at this stage. A decision as to the need for these studies would depend on numerous factors such as the type of neurotoxic effect, the potential of the chemical to accumulate, the use of the chemical, and the potential human exposure. Advanced characterisation may be appropriate if Tier 1 and Tier 2 studies are inadequate for risk assessment.

SECTION 1 INTRODUCTION

There has been increased public and scientific awareness in recent years of the potential for adverse effects of chemicals on the nervous system. Implications of permanent damage to the nervous system after exposure in the work-place, environment or during development are of particular concern. A variety of chemicals have been investigated for potential neurotoxicity; examples include solvents (Errebo-Knudsen and Olsen, 1986), pesticides (Narahashi, 1979), drugs (Allen and Young, 1978; Langston *et al*, 1983; Morrow and Routledge, 1988) and intentional and non-intentional food additives (Mahler, 1986; Janssen and Van der Heyden, 1988; Tryphonas and Iverson, 1990).

Potential damage to the nervous system is difficult to assess because of the wide variations in normal function of the nervous system as well as its plasticity, residual capacity and compensatory mechanisms (WHO, 1986). Furthermore, there may be problems in the interpretation and assessment of the relevance of the results from certain tests.

The objective of the Task Force on Neurotoxicity was to evaluate the methods for defining the neurotoxic potential of chemicals in order to provide adequate safeguards for human health.

The Task Force was given the following Terms of Reference:

- review the relevance of existing guidelines and test methods for the evaluation of neurotoxic potential of chemicals;
- recommend a practical testing strategy including details of the test methods to be employed for the evaluation of the neurotoxic potential of chemicals.

To assist the reader an explanation of the specialist terminolgy used in the field of neurotoxicity is to be found in Appendix A.

SECTION 2 BACKGROUND

The ultimate objective of any toxicity study of a chemical, whether intended as a drug, pesticide, industrial chemical or other purpose, is to provide information that will enable the safe use of the chemical. All chemicals can produce injury or death under certain exposure conditions, but the risk usually can be minimised by limiting the dose or exposure. The process of safety evaluation comprises hazard identification, hazard assessment and risk assessment. Hazard identification is the qualitative identification of the hazards which are inherent in a substance. Hazard assessment determines the potential of a chemical to cause adverse effects at a certain exposure level by identifying target organs, dose-response relationship, determining no-observed- effect levels (NOEL) and examining variations in species sensitivity. Risk assessment estimates the probability of the occurrence of an adverse effect (in man) as a result of exposure to a chemical (Appendix A).

Although the principles of hazard and risk assessment are identical for all types of chemicals, risk management may vary considerably for different types of chemicals. Thus, the safety factors that determine "safe" levels of exposure or administration of a substance vary with the intended usage. For example, a drug which is to be administered for extended periods may have a much larger safety factor applied for adverse health effects than one which is to be used occasionally or in more life-threatening conditions such as during cancer therapy. Similarly, different safety factors are applied for pesticides and industrial chemicals. A chemical which is to be used on a very small scale in specifically defined conditions may have a smaller safety factor than a chemical which is to be incorporated into food packaging.

Toxicity tests in laboratory animals are a major component of hazard identification and risk assessment. Standard toxicity studies with rats, mice, rabbits and dogs provide many opportunities to identify treatment-related effects and target organs in all organ systems (e.g. digestive, cardiovascular, urinary or nervous systems). Standard toxicity studies are reviewed in this monograph to emphasise the neurotoxicity data which can be obtained from these studies.

Neurotoxicology is a relatively new branch of toxicology and relies on a number of disciplines including psychology, neurology, physiology, pharmacology, biochemistry and pathology. Each discipline has a range of methods, with varying complexity, specificity and applicability for investigating neurotoxic effects of chemicals. The choice of methods frequently depends upon the investigators' experience with a particular test as well as cost and time factors. The methods for a particular toxicological assessment should depend upon the questions to be answered.

Evaluation of the neurotoxic potential of a chemical should include descriptions of functional and morphologic effects as well as the determination of the dose response, time course, reversibility of effects and the NOEL. Categorisation of a substance as a direct or indirect neurotoxicant is critical

for scientific and regulatory purposes. Also, investigation of the mechanism, whilst not essential, may provide additional information useful to the risk assessment process.

Few countries or organisations have regulatory requirements for specialised neurotoxicity tests of new or existing chemicals with the exception of the specific tests for organophosphate chemicals (organophosphate-induced delayed neurotoxicity [OPIDN] in the hen). Most regulatory authorities rely upon the extensive range of "standard" toxicity studies in animals to indicate those chemicals which are of concern because of potential neurotoxic activity.

A number of countries and organisations have prepared or are considering neurotoxicity test guidelines (e.g., U.S. Environmental Protection Agency [EPA, FIFRA, 1991], the International Programme on Chemical Safety of the World Health Organization [IPCS-WHO] and the Organization for Economic Co-operation and Development [OECD]). Details are to be found in Appendix B. Some of the recommended tests are neither validated nor suited to the purpose of hazard identification. In addition, the indicators or "triggers" for undertaking some of the rather specialised tests are not clear.

The Task Force reviewed existing and proposed regulatory test guidelines as well as other methods for the evaluation of potential neurotoxicity. Methods that evaluate human exposure and/or response rather than neurotoxic potential are excluded from the review. The Task Force subsequently developed a test strategy for the evaluation of potential neurotoxicity.

SECTION 3 UNDERSTANDING AND DEFINING NEUROTOXICITY

A simplistic definition of neurotoxicity (or a neurotoxic effect) is an adverse change in the structure or function of the nervous system that results from exposure to a chemical substance. A recent publication (OTA, 1990) cautions that the definition of neurotoxicity "hinges on interpretation of the word 'adverse,' and there is disagreement among scientists as to what constitutes 'adverse change.'"

Interpretation of the results from neurotoxicity studies, in terms of potential human health effects, requires an understanding of several significant issues:-

- What constitutes an adverse effect on the nervous system?
- What distinction should be made between transient effects and persistent effects?
- What is the significance of direct as opposed to indirect effects on the nervous system?
- When should a compound be considered a neurotoxic substance?

3.1. ADVERSE EFFECTS

A judgement as to what constitutes an adverse effect on the nervous system depends primarily on the nature of the change (morphological, neurochemical, neurological, or behavioural), the degree of change and whether the effect is transitory or persistent.

A reasonable definition of an adverse effect is any treatment-related change which interferes with normal function and compromises adaptation to the environment.

The term "adverse" should suggest the concept of disease and implies that the change interferes with normal function (e.g., the change is maladaptive to the individual). Adverse effects should be considered only in a toxicological sense rather than as any unwanted effect. Effects without recognised maladaptive consequences may have beneficial, indifferent or unknown consequences, but are not necessarily adverse.

Most morphological changes such as neuronopathy, axonopathy or myelinopathy would be considered adverse even if the changes were mild and/or transitory. On the other hand, transitory hypertrophy of astrocytes could be viewed as an adaptive, physiological response. A more complicated example is the transitory reduction in the number of dendritic spines on pyramidal neurons in rats exposed *in utero* to ethanol (Ferrer *et al*, 1988). Although a

reduction in the number of dendritic spines in 15-day old rats was followed by morphological recovery at 90 days of age, the results would not necessarily prove functional recovery.

Neurochemical changes in the nervous system of experimental animals should not necessarily be interpreted as adverse without additional correlative information. For example, gerbils exposed to near-lethal levels of dichloromethane (DCM) for 2 months and examined 4 months post-exposure, were reported to have changes in brain levels of DNA as well as glial fibrillary acidic protein (GFAP) and S-100 protein (Rosengren *et al*, 1986). A subsequent study was conducted in order to determine if exposure to DCM was associated with any toxicologically adverse effects on the nervous system (Mattsson *et al*, 1990a). Detailed clinical, neurofunctional and neuropathological examinations of rats exposed to high levels of DCM for 13 weeks did not reveal any persistent post-exposure, treatment-related effects and call into question the relevance of the changes reported in the initial study.

Although neurotypic and gliotypic proteins have been proposed as biochemical markers of neurotoxicity (O'Callaghan, 1988), the EPA Science Advisory Panel concluded that radio-immunoassay measurement of GFAP in the brain is not sufficiently validated as an indicator of neurotoxicity to be incorporated in test guidelines (SAP, 1989). Likewise, there is considerable doubt about the validity of plasma and red cell cholinesterase inhibition as an indicator of neurotoxicity (SAB/SAP, 1990) since these biochemical measures are not correlated with recognised adverse effects. The Panel recommended that adverse effects on the nervous system should be defined on the basis of functional measures (behavioural, electrophysiological) accompanied by morphological indices such as histochemical techniques.

3.2. TRANSIENT VERSUS PERSISTENT EFFECTS

Transient, acute neurological effects of compounds should be distinguished from permanent or chronic effects. Many chemicals can have non-specific pharmacological effects on the nervous system at high exposure levels, but these effects are not relevant for prediction of neurotoxic effects at occupational or environmental exposure levels.

The potential consequences of neuropharmacologic effects vary significantly. Similar to the situation with anaesthetics, some reversible functional or behavioural changes that are not associated with permanent morphological alterations would not be considered adverse in themselves. On the other hand, pharmacologic effects in man may induce adverse consequences by a reduction in vigilance which possibly could result in accidents. Furthermore, chemicals such as organophosphates clearly have adverse acute neurotoxic effects because of their pharmacologic mechanism of action.

Dichloromethane (DCM) is an example of a substance with transient neuropharmacological effects, but no confirmed neurotoxic effects (Mattsson *et al*, 1990a). DCM causes central nervous system depression at relatively high concentrations and also alters neurophysiological functions of rats during exposure to 2,000-5,000 ppm DCM. Post-exposure functional tests (observational battery, grip strength, and evoked potentials) along with detailed pathological examinations of rats exposed to as much as 2,000 ppm DCM for 6 h/d, 5 d/wk for 13 weeks did not reveal any treatment-related changes. Thus, even though DCM does have sedative and anaesthetic properties at relatively high concentrations, brain injury appears unlikely.

3.3. DIRECT VERSUS INDIRECT EFFECTS

Another important concept in the practical definition of neurotoxicity is the significant difference between direct (primary) as opposed to indirect (secondary) effects on the nervous system. A wide variety of disease conditions such as nutritional and metabolic disorders, diabetes mellitus, liver disease and kidney disease may have secondary effects on the function and structure of the nervous system, such as encephalopathy or polyneuropathy (Robbins and Cotran, 1979). Exposures which do not cause primary toxicity in the liver or kidney, for example, would not result in any secondary effects on the nervous system. Thus, differentiation between direct and indirect effects of a substance on the nervous system is critical for scientific and regulatory considerations.

The indirect effect of substances on the nervous system is a major concern for the interpretation of acute toxicity studies which are conducted at very high dose levels. Substances which are not directly toxic to the nervous system can cause signs such as convulsions, tremors or ataxia at toxic or lethal dose levels. Thus, essentially all substances could be classified as "neurotoxic" since all chemicals are toxic at some dose level and will induce some behavioural change at these very high dose levels. These concerns were summarised by the EPA's Science Advisory Panel (SAP, 1989) when they stated "The Panel has serious reservations about the validity of neurotoxicity studies in which the high-dose level results in gross changes which exceed the MTD [maximum tolerated dose] or in which the normal metabolic processes of the body are severely compromised."

The interpretation of neurofunctional data from routine toxicity studies may be confounded by systemic toxicity. Gerber and O'Shaughnessy (1986) evaluated the specificity of several functional and behavioural tests for nervous system toxicity. The results indicated that impairment of organs other than the nervous system as well as reduced food and water intake can mimic the behavioural effects of standard neurotoxic agents. In fact, Gerber and O'Shaughnessy (1986) suggest that "before it can be concluded that a compound is

neurotoxic on the basis of behavioural test results, it must be ascertained that non-neural organs have not been damaged by the test compound, and that food and water consumption have not been severely decreased."

Further studies of dietary restriction in rats revealed significant effects on physical, behavioural and neurophysiological parameters after 4 weeks of dietary restriction (Albee *et al*, 1987). Thus, the relevance of neurofunctional changes is obscure in the presence of general toxicity and in the absence of neuropathological changes (Mattsson *et al*, 1989).

Interpretation of results from motor activity tests (cf. Appendix C.1.2) is difficult especially in terms of direct versus indirect effects on the nervous system. A dose-related change in motor activity reflects an effect on the nervous system only in the absence of general toxicity; additional information is required to determine that such an effect actually is adverse (Maurissen and Mattsson, 1989). Conclusions about nervous system involvement based on motor activity can be made only by exclusion and thus, interpretation should be limited to a dose range not associated with general toxicity.

Neurotoxicity should imply a direct effect on the nervous system while behavioural or neurofunctional effects are terms that indicate a more general functional change, whether or not such an effect originates in the nervous system (Maurissen and Mattsson, 1989). Therefore, indirect effects that are detected by neurofunctional tests such as clinical observations, motor activity and other behavioural tests, clearly should be distinguished from direct neurotoxic effects.

3.4. DEFINITION OF A NEUROTOXIC SUBSTANCE

Designation of a substance as neurotoxic should be reserved for those xenobiotic compounds or their metabolites that produce adverse effects as a result of direct interaction with the nervous system. Substances which are not directly toxic to the nervous system, but result in neurobehavioural signs as a result of damage to other organ systems, should not be considered as neurotoxicants.

An assessment of potential neurotoxicity should be based on a number of different parameters that are derived from a variety of toxicological tests at relevant dose levels. A combination of functional and morphological tests enhances the ability to discover neurotoxicity.

Inappropriate categorisation of a substance as "neurotoxic" could be best avoided by a clear understanding of the general toxicity of a chemical prior to specialised neurotoxicity studies.

Subsequent specialised tests for neurotoxicity should only be conducted at dose levels where no adverse general effects were detected with routine toxicity studies. Thus, a critical point in the evaluation of potential neurotoxicity is the threshold dose for neurotoxicity; substances with neurotoxic effects at levels which are less than the NOEL for other toxic effects would be of concern.

Criteria for categorisation of a substance as a human neurotoxicant were proposed for solvents by Spencer and Schaumburg (1985). Three questions must be answered affirmatively before a solvent is accepted as a human neurotoxicant:

- does the substance or mixture produce a consistent pattern of neurological dysfunction in man;
- can this entity be induced in animals under comparable exposure conditions;
- are there reproducible lesions in the nervous system or special sense organs of exposed human beings and/or animals, and do these abnormalities satisfactorily account for the neurobehavioural dysfunction?

These criteria also are applicable to chemicals other than solvents and would be useful in the categorisation of substances in regard to neurotoxicity for man.

A substance could be considered a 'possible human neurotoxicant' if animal toxicity studies reveal limited evidence of direct, adverse, persistent effects on the nervous system. Indirect effects on the nervous system that clearly are secondary to systemic toxicity would not be a basis for even this category.

When animal neurotoxicity studies provide sufficient evidence of direct, adverse, persistent effects on the nervous system and are likely to be consistent across species, the substance could then be considered as a 'probable human neurotoxicant'.

Finally, fulfilment of criteria similar to those of Spencer and Schaumburg should be applied in order to categorise a substance as a 'known human neurotoxicant'.

SECTION 4 REVIEW OF CURRENT TEST METHODS

The design of neurotoxicity studies should incorporate clear objectives and produce interpretable data. The World Health Organisation (WHO, 1986) defined the objectives of neurotoxicity testing as:

- identify whether the nervous system is altered by the toxicant (detection);
- characterise nervous system alterations associated with exposure;
- ascertain whether the nervous system is the primary target for the chemical;
- determine dose- and time-effect relationships in order to establish a no-observed-adverse-effect level.

These objectives translate into a series of questions about the toxicity of a chemical that may be answered with standard toxicity tests as an initial screen and/or more specialised neurotoxicity studies.

4.1. STANDARD TOXICITY STUDIES

The term "standard toxicity studies" in this monograph refers to the toxicity test guidelines of OECD (1981), EEC (1983), EPA/TSCA (1983-84), EPA/FIFRA (1991) and Japan/MAFF (1985). Relevant information concerning potential target organs including the nervous system can be obtained from acute (single dose), subacute, subchronic and chronic toxicity studies and reproduction studies.

4.1.1. Information on Potential Neurotoxicity from Standard Toxicity Studies

Standard toxicity studies are important in the assessment of potential neurotoxicity of a compound because these studies are conducted at relatively high doses, with different durations and routes of administration as well as with several species of animal. Metabolism and pharmacokinetic data often are developed in support of the standard toxicity studies for many compounds. Standard toxicity studies evaluate functional, behavioural and morphological endpoints for the nervous system which may give preliminary or definite indications of the neurotoxicity of xenobiotics (Steinberg, 1987).

The variety of dosing regimens for standard studies is important because some chemicals induce effects after single exposure (e.g., trimethyltin, Hagen *et al*, 1988; organophosphates, Abou-Donia and Lapadula, 1990) whereas others require repeated exposure (e.g., acrylamide, Bogo *et al.* 1981). Not only is the duration of exposure

important but, for some chemicals, the exposure pattern is important in determining whether or not the material is neurotoxic. For example, exposure to hexane at 1,000 ppm 24 h/d, 5 d/wk for 11 weeks produced clear and long-lasting neurotoxicity but exposure at 24,000 or 48,000 ppm for brief (10 minutes) periods 6 or 12 times per day (i.e., equivalent or higher total exposure) produced only slight effects (Pryor *et al*, 1982).

Contribution from clinical observations. Clinical observations included in standard toxicity protocols usually are obtained by cage-side monitoring of animals, as well as during handling at the time of dosing or body weight determination. Clinical observations may indicate changes in motor function (e.g., disturbances of gait, abnormal posture or muscle tone), arousal state (e.g., hyperactivity, apathy or lethargy), psychological state (stereotypies, aggression, biting, licking, self-mutilation) or indications of pharmacological effects (sedation, anaesthesia).

Indirect evidence of neurotoxicity from standard toxicity studies may be suggested by the general physiological state of the test animals. The integrity of the autonomic nervous system can be assessed with observations of specific functions such as salivation, lacrimation, urination or defecation.

Clinical observation of adults or pups in standard reproduction studies may give an indication of altered neuromotor functions or arousal states that may be affected by developmental neurotoxicants. Successful mating, delivery and rearing of pups depend on normal behaviour and appropriate function of multiple organ systems including the nervous system. Also, physical and functional landmarks of pups are sensitive parameters of development. Furthermore, reproduction by F1 animals provides additional information on nervous system development.

Contribution from Morphological Examinations. Standard toxicity studies generally include gross examination of most organs and tissues, measurement of the weight of organs including the brain, and histopathological evaluation of brain, spinal cord, peripheral nerve, muscle, eyes as well as many other tissues. Thus, a broad range of cellular elements and functional entities which comprise the nervous system is evaluated by standard toxicity studies.

Histopathology in standard toxicity studies often includes examinations of brain structures that are related to specific types of behaviour. Examples of such structures include:

- the hippocampus, which is important for memory;

- components of the limbic system, which are responsible for emotion;
- the hypothalamus, which is associated with autonomic integration and control of the endocrine system.

Histopathological examination of all nervous tissue is impracticable. Representative samples of nervous tissues are adequate (Hirano and Llena, 1980; Thomas, 1980). Furthermore, since practically all tissues include nervous tissue, routine microscopic sections in standard toxicity studies (e.g., skin, intestine and muscle) also contribute to the comprehensive examination of the nervous system. In addition, since the functional and structural integrity of effector organs may be altered by changes in the normal control by the nervous system, histopathological examination of tissues such as muscle, exocrine glands, viscera, reproductive organs, and, in particular, sensory organs and endocrine glands may give indirect indications of neurotoxic effects.

4.1.2. Examples of Neurotoxicity Detected with Standard Toxicity Studies

This section includes examples of neurotoxic chemicals with special emphasis on those which illustrate the effectiveness of standard toxicity studies for the detection of nervous system effects. The examples are grouped according to a classification of neurotoxic diseases (Spencer and Schaumburg, 1980a) which are associated with similar patterns of functional impairment and morphological changes.

Neuronopathy. Neuronopathies can be induced with a variety of substances including methylmercury, bilirubin, acetylethyl tetramethyl tetralin, chloroquin, diphenylhydantoin, aluminium, colchicine, vincristine and vinblastine (Hirano and Llena, 1980). Morphologically detectable lesions and associated clinical signs vary with the target site and the progress of the disease. Vacuolation and neuronal degeneration are among the earliest indications, followed by neuronal necrosis and cell loss as well as by secondary axonal changes. Toxic neuronopathies with central nervous system involvement usually are characterised by clinical signs that include behavioural changes, disturbed motor function and changed regulation of vital physiological functions.

Morphological lesions in peripheral neuronopathies are found in dorsal root, trigeminal and autonomic ganglia. Depending on the underlying toxic mechanism, the earliest detectable changes may consist of displacement of the nucleus and rearrangement of Nissl-substance (e.g., pyridoxine; Krinke and Fitzgerald, 1988) or nuclear pyknosis and karyolysis with subsequent necrosis of affected neurons (e.g., doxorubicin; Jortner,

1988). More advanced stages of the disease are characterised by secondary axonal degeneration or neuronal loss. This type of neuronal and axonal damage is associated with prominent clinical signs such as ataxia, incoordination and tetraparesis; paresis may be more prominent in hind-limbs.

Neuronopathies have been detected by standard toxicity studies that range from acute to chronic in duration. For example, a battery of acute and subacute studies evaluated an acetylenic compound (5,7,11-dodecatriyn-1-ol) as part of an ongoing research and development programme (Gad *et al*, 1988). Clinical signs of neuromuscular involvement (including hind-limb paralysis) were detected in guinea pigs during the course of a photosensitisation study; supplementary evaluations revealed bilateral necrosis of the medulla oblongata. A similar lesion was found in rats after a single oral dose of the test material at 0.25 ml/kg bw as well as in rats dosed daily for 2 weeks with 0.03 ml/kg bw.

Standard subacute and subchronic inhalation toxicity studies in rats and rabbits demonstrated the neurotoxic potential of the fumigant sulphuryl fluoride (Eisenbrandt and Nitschke, 1989). Two-week and 13-week exposures of rabbits to 600 ppm sulphuryl fluoride resulted in hyperactivity and convulsions while exposures to 300 or 600 ppm produced vacuolation and/or malacia in the cerebrum; one rabbit exposed to 100 ppm also had cerebral vacuolation. Although neurotoxicity was not present in the 2-week rat study (the target organ was kidney), the 13-week rat study demonstrated cerebral vacuolation in rats exposed to 300 ppm sulphuryl fluoride.

A subchronic rat study conducted by the US National Toxicology Program (NTP) revealed the neurotoxic potential of tris(2-chloroethyl)phosphate (Matthews *et al*, 1990). Routine clinical observations detected convulsions, salivation, gasping and lack of coordination. Also, standard histopathological evaluation indicated neuronal loss within the hippocampus.

The fragrance and flavouring agent benzaldehyde produced neurotoxic effects in rats that included hyperexcitability, tremors and inactivity as well as necrotic and degenerative lesions in cerebellum and hippocampal regions of the brain. The neurotoxicity was detected by a standard subchronic toxicity study at NTP (Kluwe *et al*, 1983).

A chronic, 2-year feeding study with methylmercury chloride was conducted in B6C3F1 mice and clearly detected neurotoxicity (Mitsumori *et al*, 1990). Mice treated with 10 ppm of the test material developed neurotoxic signs characterised by posterior

paralysis. Standard pathology procedures (immersion fixation, paraffin embedding and haematoxylin and eosin stains) revealed toxic encephalopathy and peripheral, sensory neuropathy. Neuronal necrosis occurred in the cerebral cortex, caudate nucleus and cerebellar granular layer. The neuropathy was characterised by degeneration of myelinated nerve fibres in the spinal dorsal nerve roots and sciatic nerves as well as neuronal loss in the spinal ganglia. Similar degeneration was present in the spinal dorsal funiculus which corresponds to the ascending pathway of the peripheral sensory nerves.

Myelinopathy. Chemicals that produce myelinopathy include triethyltin (Watanabe, 1980), hexachlorophene (Towfighi, 1980), 2-chloro-2,4-dinitro 5,6-di(trifluoromethyl)diphenylamine (CDTD; Lock *et al*, 1981), isonicotinic acid hydrazide (Blakemore, 1980) and cuprizone (Politis *et al*, 1980).

Clinical signs associated with myelinopathy are lethargy and gait abnormalities due to muscle weakness; at high dosages paralysis, tremor and convulsions can be observed. Gross morphologic changes such as brain oedema, hydrocephalus and increased brain weight indicate the presence of neurotoxic effects. Diffuse spongiosis within the white matter of cerebrum, cerebellum and/or spinal cord is the most prominent microscopic alteration following acute (single) exposure of sufficiently high dosages. Continuous exposure may lead to demyelination and secondary axonal damage.

There are numerous examples of chemically-induced myelinopathies that have been detected by standard toxicity studies. Indications of neurotoxic effects have been derived from routine toxicity studies with isonicotinic acid hydrazide (Noel *et al*, 1967), triethyltin (Stoner *et al*, 1955; Magee *et al*, 1957) and CDTD (Lock *et al*, 1981).

Dichloroacetate is a by-product of the chlorination process for disinfection of drinking water; the diisopropylammonium salt of this compound has been used for the therapy of diabetes, hyperlacticemia and hypercholesterolemia. Standard subchronic (90-day) toxicity studies in rats and dogs have been conducted for the purpose of risk assessment with regard to human exposure (Katz *et al*, 1981; Bhat *et al*, 1991; Cicmanec *et al*, 1991). The results indicated unequivocal evidence of myelinopathy as demonstrated by the respective clinical signs (hind-limb weakness and/or paralysis) and histopathological lesions in the brain (vacuolation of myelinated white tracts in the cerebrum, cerebellum and spinal cord).

Gavage studies of glycidol detected effects on the brain of rats and mice with routine pathology procedures (NTP, 1990). Lesions were present only in animals dosed with

lethal or near-lethal concentrations. Focal demyelination was observed in the medulla and thalamus in all female mice dosed with 300 mg/kg bw in the 16-day study. Demyelination of the brain was present in male and female mice that received 150 or 300 mg/kg bw in the 13-week study; rats that were dosed with 400 mg/kg bw in the 13-week study had necrosis of the cerebellum and demyelination in the medulla. On the other hand, 2-year studies of glycidol in rats (37,5 and 75 mg/kg bw) and mice (25 and 50 mg/kg bw did not reveal any treatment-related effects on the nervous system.

The chronic (1-year) toxicity of vigabatrin, a GABA-transaminase inhibitor, was evaluated in rats and dogs with standard protocols (Gibson *et al*, 1990). Dietary administration of vigabatrin resulted in convulsions in rats beginning after 3-4 months administration. Intramyelinic oedema (microvacuolation) was detected in the white matter of the cerebellum, reticular formation and optic tracts in the rats as well as the columns of fornix and optic tract in the dogs.

A chronic (9-month) toxicity study in dogs with the anticancer agent tegafururacil produced marked clinical signs such as excitement, hyperactivity, hyperrespiration, hyperesthesia, convulsions and gasping. Cerebral vacuolation was observed as a morphological correlate predominantly in dogs which had died prematurely (Yamashita *et al*, 1988).

Axonopathy. There are several chemicals known to induce selective degeneration of distal axons within the central and/or peripheral nervous system following systemic exposure. The most comprehensive examples are acrylamide (Le Quesne, 1980), carbon disulfide (Seppalainen and Haltia, 1980), clioquinol (Krinke *et al*, 1979), doxorubicin (Cho *et al*, 1980), the hexacarbons n-hexane, methyl-n-butyl ketone and 2,5-hexandione (Spencer *et al*, 1980b), methyl mercury (Mitsumori *et al*, 1990) and 3'3'iminodipropionitrile (IDPN), a prototypic compound for the induction of proximal axonopathy in rats (Griffin and Price, 1980).

Compounds that induce distal axonopathies typically produce a similar pattern of functional impairment and morphological damage. Neuromuscular signs such as weakness, abnormal gait, incoordination and ataxia are the most prominent symptoms during the early stages of distal axonopathies. Sensory nerve fibers are usually more susceptible than motor fibres, but the effects may be overlooked due to the absence of prominent clinical signs associated with most sensory neuronopathies (e.g., toluene ototoxicity). The morphological correlate of these clinical signs includes axonal swellings predominantly in peripheral nerves and long tracts of the spinal cord.

Following prolonged exposure, denervation atrophy of skeletal muscles can be observed as a secondary effect.

Toxic distal axonopathies can be detected with standard subchronic or chronic protocols. A study by Schulze and Boysen (1991) demonstrated manifestations of acrylamide neurotoxicity in rats after 5 weeks of intragastric dosing at 30 mg/kg bw/day. Although specific behavioural measures and histopathological procedures were included in this study, the description of changes suggests that standard parameters such as cage-side observations and routine histopathology of nervous tissues are suitable for detection of acrylamide neurotoxicity. For example, chronic toxicity studies in rats exposed to acrylamide in drinking water revealed degeneration of peripheral nerves in rats that received only 2.0 mg/kg bw/day (Johnson *et al*, 1986). The degeneration was observed microscopically in the tibial nerve of rats at 12, 18 and 24 months of treatment.

4.2. NEUROTOXICITY TEST METHODS

Specific methods for neurotoxicological tests are presented in this section. The four main subjects are behaviour, electrophysiology, neurochemistry and neuropathology. Additional sections are included on tests for developmental neurotoxicity and the use of *in vitro* or other alternative test methods. Additional details for these methods are presented in Appendix C.

4.2.1. Behavioural Tests

Behavioural changes following exposure to a neurotoxic chemical can be sensitive indicators of disturbed function of the nervous system since they may be observed earlier and/or at doses lower than demonstrable clinical symptoms or structural lesions (Rice, 1980; Alder and Zbinden, 1977, 1983; Walsh and Chrobak, 1987; Broxup *et al*, 1989a; Schulze and Boysen, 1991). Due to the functional reserve capacity of the nervous system, there is the possibility of an animal suffering some structural loss but still remaining functionally normal (Mitchell and Tilson, 1982). More important is the lack of specificity of most behaviour tests. Not all behavioural changes necessarily represent the specific action of a chemical on the nervous system and many behavioural tests are affected by changes in non-neural organs (Gerber and O'Shaughnessy, 1986; Rice, 1990) as well as by dietary restriction (Albee *et al*, 1987), hormonal state (Robbins, 1977), fatigue (Bogo *et al*, 1981), motivation (Cooper, 1981) or age (Soffie and Bronchart, 1988). Some behaviours are affected by housing conditions and, thus, may not be apparent in a particular test. For example, one of the characteristic signs of trimethyltin neurotoxicity in rats is aggression which is observed as sparring between cage-mates (Dyer and Howell,

1982); aggression is not seen when animals are singly housed, rather self-mutilation is observed instead (Bouldin *et al.* 1981).

The choice of behavioural test depends upon the purpose of the study; some tests may be simple to perform, but lack sensitivity, whereas others are much more sensitive, but are complex and time consuming. On the other hand, a complex test is not necessarily a sensitive one. For example, a comparison of the relative sensitivity of a functional observational battery (FOB), motor activity (MA) and schedule-controlled operant behaviour (SCOB) indicated that the FOB was as sensitive or more sensitive than MA or SCOB in detecting treatment-related effects (Moser and MacPhail, 1990).

Observation of behaviour can be incorporated into standard test protocols. Typically, observation of the animal for signs such as lethargy or hyperactivity, piloerection, salivation, convulsions, ataxia, abnormal gait, and tremor is an integral part of standard toxicity studies. Thus, any assessment of behavioural neurotoxicity should begin with simple and rapid tests to determine if behavioural effects are present and then proceed, if necessary, with tests of increasing complexity and duration.

Rodents (rat or mouse) are normally the species of choice for screening procedures. Birds and cats, however, may be a better choice for determining toxicity of the visual system (Evans, 1982; Rice, 1990). More complex and sophisticated procedures such as tests of fine motor control or some kinds of cognitive testing are usually best done in the primate (Rice, 1980).

4.2.2. Electrophysiological Techniques

Neurophysiological techniques measure the electrical potentials of impulse transmission in the nervous system. The potentials reflect the functioning of the neuron or neurons that generated them. These potentials are usually measured by placing electrodes in, on or near the neural tissue of interest. The electrical signals recorded by the electrodes are amplified, filtered and passed to a data acquisition device. Depending on the size of the electrode, the techniques may be used to measure the function of single cells (or parts of cells), or the summed activity of thousands of cells in specific neural systems. Electrical potentials may be recorded in specific areas of the central or peripheral nervous system *in vivo* or they even can be recorded from *in vitro* preparations.

Electrophysiological methods offer many advantages to the neurotoxicologist in the study of potentially neurotoxic chemicals. These include: the ease with which most biopotentials can be measured in experimental animals; the relative ease with which the data can be analysed, quantified and standardised; and the large amount of electrophysiological data

that can be collected quickly. Some techniques are also non-invasive and allow monitoring of progression and/or recovery of functional disturbance.

Electrophysiological techniques are commonly used by neurophysiologists and clinical neurologists. The procedures have been presented in the literature (Thompson and Patterson, 1974; Barber, 1980) and provide a large body of information against which test data can be compared. In addition, the biological basis of electrophysiology allows the techniques to be readily applied across species, including man (Seppalainen, 1975; Rebert, 1983; Dyer, 1985; Arezzo *et al*, 1985; Mattsson and Albee, 1988; Mattsson *et al*, 1989). Most electro-physiological data are easily extrapolated to man since these data are familiar to the medical community (Mattsson and Albee, 1988).

The electrophysiological method for a particular experiment must be appropriate to the question being asked. If one is interested in overt changes, then macroelectrode procedures, such as EEG, may be adequate (OTA, 1990). More specific questions such as whether the chemical acts on pre-synaptic receptors, specific ion channels, or sensory rather than motor nerves, demand more sophisticated experimental procedures. The latter techniques may provide specific information on the mechanism of neurotoxicity of a particular chemical. Nevertheless, a multi-disciplinary approach will facilitate a better understanding of the effects of chemicals on the nervous system (WHO, 1986; OTA, 1990).

4.2.3. Neurochemical Tests

Various neurochemical methods have been designed to assess mechanisms and effects of psychopharmacological agents and are used increasingly to investigate mechanisms of neurotoxicity. Some neurotoxic mechanisms for specific chemicals are listed in Appendix C Table C-3. The nervous system is comprised of several cell types and multiple transmitter systems. The different cell types make unique contributions to neural function and differ in their sensitivity to chemicals. The functional integrity of such a complex system cannot be comprehensively assessed by measuring only a few biochemical parameters.

Unlike blood chemical parameters which are used to assess systemic organ damage, neurochemical measurements in general are performed on nerve tissue. These can be parts of peripheral nerves, the entire brain of animals, distinct brain structures obtained by dissecting whole brains, slices of whole brain or of particular brain structures, neurons or glial cells cultured *in vitro* or isolated from brain. To increase the sensitivity of neurochemical methods, cells can be fractionated and particular cell organelles separated.

Since neuronal lesions generally are limited to specific areas of the brain and often to specific types of neurons, the sensitivity of neurochemical measurements decreases with increasing volume of nerve tissue in a single assay. In contrast, the chance of missing an effect increases with decreasing total volume of tissue. In addition, the relationship between nervous system function and observations made in neural tissue extract in a test tube is somewhat tenuous because the concentration of many endogenous substances or the activity of enzymes may change rapidly after death. Further problems arise due to the tendency of the nervous system to compensate for neuronal loss, for example, by increasing turnover rate of transmitters or by up- or down-regulation of receptors (Cooper *et al*, 1986).

Based on the above considerations, neurochemical methods are unsuitable as routine screens for neurotoxic effects. Too many different parameters have to be measured to assess comprehensively the neurotoxic potential of a given compound. In cases where the neurotoxic mechanism of a compound is known, a few critical parameters related to its neurotoxic effect can be measured to screen rapidly structural analogues for that specific neurotoxic mechanism.

Because of their specificity neurochemical methods have been used successfully to elucidate possible neurotoxic mechanisms of chemicals. These methods further the understanding of neurotoxic mechanisms and thereby make an important contribution to the current knowledge of neurotoxicology.

An interesting and promising approach to overcome some of the problems related to neurochemical assays is the combination of neurochemical determinations and histopathology (histochemical staining techniques). Such techniques are specific, sensitive and have the advantage of showing the topographic distribution of any findings (Krinke and Hess, 1981).

4.2.4. Neuropathological Methods

The morphological complexity of the developing and mature central nervous systems (CNS) and peripheral nervous systems (PNS) must be taken into account in the application of histopathological techniques for the assessment of the neurotoxic potential of chemicals (Altman, 1973, 1976; Palay and Chan-Palay, 1974; Gardner, 1975; Peters *et al*, 1976). Because of the highly specialised structure of the vasculature and associated blood-brain and blood-nerve barriers (Reese and Karnovsky, 1967; Mizisin *et al*, 1990), the CNS ventricular system, the neuron and supporting glial network, as well as the organs of special sense, there is potential for a variety of pathological responses to toxic

injury caused by chemicals (WHO, 1986). Perhaps more than for any other organ, the choice of experimental techniques and the actual evaluation of the range of dysgenetic and degenerative morphologic changes throughout the nervous system depends on a high degree of neuropathological training and experience (Cavanagh, 1973; Spencer and Schaumburg, 1976; Hirano and Llena, 1980; Suzuki, 1980; Rodier, 1990).

There is a plethora of possible central and peripheral neuropathological responses to a neurotoxic chemical (Appendix D). The many factors affecting the ability to detect these responses have been reviewed recently (Spencer and Schaumburg, 1980b; WHO, 1986; Krinke, 1989).

Although many neurotoxicological entities in man can be readily reproduced in the rat (Spencer and Schaumburg, 1980b), certain neuropathological effects, such as those associated with tri-o-cresyl phosphate intoxication or high doses of lovastatin, are not always manifested in this species (Berry *et al*, 1988; Somkuti *et al*, 1988). Age of the test animal also may affect detection of a change, certain responses being most marked at a particular stage of development, for example, haemorrhagic encephalopathy caused by tunicamycins or corynetoxins in the immature rat (Berry and Vogel, 1982; Finnie and O'Shea, 1988). Interpretation of pathological changes may be confounded by spontaneous background lesions in subchronic (Eisenbrandt *et al*, 1990) and chronic studies. For example, neurotoxic peripheral neuropathy in the rat may be complicated by spinal radiculoneuropathy which increases in incidence and severity with age (Burek *et al*, 1976; Krinke, 1983).

The distribution, morphology and time course (spatio-temporal pattern) of a lesion is an essential consideration for its detection. Most lesions are assessable by standard semi-quantitative pathological evaluation. Nevertheless, certain pathological changes, particularly those associated with neurodevelopmental toxicants or chemicals causing low grade peripheral neuropathy, may only be detected using either morphometry (Rodier, 1979, 1990; Broxup *et al*, 1990), or special techniques to demonstrate subtle changes, such as those found in neurites of rats exposed prenatally to lead or ethanol (Averill and Needleman, 1980; West and Hodges-Savola, 1983). Certain neuropathological effects, such as those reported with amoscanate, pyridoxine or tunicamycin, characteristically occur within a few days of exposure (Krinke *et al*, 1983, 1985; Finnie and O'Shea, 1988). With other compounds, such as high-dosed lovastatin in dogs, clinical evidence of neurotoxicity may not develop for several weeks (Berry *et al*, 1988).

Careful consideration must be given as to the purpose of a study when choosing neuropathological techniques. When chemicals are initially tested for toxicity, effective use

of animals and resource involves choice of techniques that allow thorough examination of the central and peripheral nervous system, but does not disrupt pathological examination of other organs. Thus, in a potential first tier of neurotoxicity screening when there has been no prior indication of any neurotoxic effect of a chemical, standard pathological methods should be incorporated. Several investigators have described Tier 1 methods in the rat based on immersion fixation of nervous tissue complemented with appropriate functional assessment (cf. 4.2.1. and 4.2.2.). When available data suggest a chemical produces neurotoxicity, second tier pathological techniques, usually incorporating perfusion fixation, may be appropriate (O'Donoghue, 1989; Mattsson *et al*, 1990b) with supplementary specialised procedures to define particular effects and to avoid misinterpretation of artefacts.

Neuropathology should be integrated with functional studies (Tilson *et al*, 1979; Johnson and Richardson 1983; WHO, 1986; O'Donoghue, 1989; Mattsson *et al*, 1989, 1990b). While neuropathology provides clearly interpretable data and high resolution (including single neurons and axons), the methods are limited to static evaluation of discrete sections. On the other hand, functional tests evaluate dynamic system functions and populations of cells; nevertheless, these tests are somewhat limited in resolution and interpretability and are subject to masking or compensation. Adequate definition of an encephalopathy or neuropathy may be enhanced by an understanding of the clinical or functional disturbance and the morphologic effect (Spencer and Schaumburg, 1980b; Dyck *et al*, 1986; Krinke 1989, Mattsson *et al*, 1989, 1990b).

There are several examples of studies that illustrate the utility of integrating morphological and functional techniques. A subchronic neurotoxicity study of the fumigant sulphuryl fluoride in rats demonstrated complementary functional and morphologic findings that would not have been detected with only one of the methods. Specifically, the functional effects (slowed visual, auditory and somatomotor evoked potentials) indicated a diffuse CNS effect which did not correlate with the localised morphologic effects in the caudate-putamen (Mattsson *et al*, 1988). The integrated approach to neurotoxicology also was applied in the pre-clinical evaluation of the cholesterol-lowering drug lovastatin (Berry *et al*, 1988; MacDonald *et al*, 1988). Standard sub-chronic studies in beagle dogs given up to 180 times the human therapeutic dose indicated correlated functional and morphologic changes related to the CNS, i.e., convulsions and multifocal ischemic necrosis.

The pathologist must always be on the alert for lesions that have undetected clinical correlates. For example, acrylamide produces prominent motor deficits that are correlated with Wallerian degeneration of peripheral nerves; however, damage to central neurons

and to the autonomic nervous system were detected only by neuropathologic examinations (Boyes and Cooper, 1981; Sterman *et al*, 1983).

4.2.5. Developmental Neurotoxicity

Many teratogens affect the nervous system (Rodier, 1990). Therefore, examination of effects on the nervous system of developing animals is an important aspect of the assessment of developmental toxicity. Exposure of developing animals to a chemical may result in quantitatively and qualitatively different effects than exposure of adult animals. Examples include ethanol (Meyer *et al*, 1990; Rees *et al*, 1990) and the relative resistance of the weanling rat to hexane neuropathy (Howd *et al*, 1983).

The stage of development of the nervous system at birth varies with different species. For example, the neonatal rat is at a stage of development most similar to that of man at the beginning of the third trimester of pregnancy (Nikimura and Shiota, 1977). A number of developmental landmarks have been defined which reflect normal development. Thus for Sprague-Dawley rats the following are relevant: pinna detachment (at day 2), primary coat of downy hair (at day 5), incisor eruption (at day 8), development of fur (at day 9), ear opening (at day 11), eye opening (at day 14), testes descent (at day 25), vaginal opening (at day 30) and balano-preputial separation (at day 44) (Alder and Zbinden, 1977). Some of these physical landmarks are closely connected to the development of the nervous system and their evaluation may give a first indication for an impaired nervous system development.

Parallel to physical development, functional development of animals also may indicate an impairment of neural function. Some of the functional landmarks for rats are: surface righting (at day 3), negative geotaxis (at day 7), disappearance of pivoting (at day 4), olfactory orientation (at day 8), hind-limb support (at day 12), auditory startle (at day 12) and mid-air righting (at day 17) (cf. Appendix C.5 and Table C-9). These functional tests are easy to conduct and can be included in routine reproductive toxicity studies.

More specific measurements of behaviour, sensory and cognitive functions such as odour or taste aversion, active and passive avoidance or motor activity can be conducted to characterise particular effects. Contrary to the simple functional tests, these more specific tests in general have to be conducted with older animals and cannot easily be integrated into standard protocols. Large test batteries have been developed for comprehensive examination of developing animals. Four major test batteries have been described (Adams, 1986):

- the Collaborative Behavioural Teratology Study Battery,

- the Cincinnati Psychoteratogenicity Screening test battery,
- the Barlow Sullivan Screening battery,
- the Japanese Battery for Behavioural Teratology Screening.

Common to all the test batteries is the preferential assessment of physical and functional landmarks. These landmarks seem to be more sensitive indicators than the more specific measures of behaviour, sensory and cognitive functions (Elsner *et al*, 1988, 1991; Elsner, 1991) and thus are valuable tools for the detection of potential developmental neurotoxicity.

Both maternal toxicity and systemic toxicity in the offspring are taken into account in the assessment of specific effects on the developing nervous system. Test substances which induce severe maternal toxicity might generate false positive results in the pups; any developmental effects, especially altered behaviour, may occur as a consequence of maternal toxicity rather than being a direct effect of the test substance on the offspring.

4.2.6. Alternative (Non-Mammalian) *In Vivo* and *In Vitro* Methods

Details of alternative *in vivo* and *in vitro* techniques are given in Appendix C, Tables C-10 and C-11.

Interest in the use of alternative *in vivo* or *in vitro* techniques in neurotoxicology is increasing partly because of the use of such techniques in other branches of toxicology, e.g., genotoxicity. No alternative *in vivo* or *in vitro* test is widely accepted as a routine pre-screening test for neurotoxicity. The difficulty of developing any *in vitro* pre-screening test to reflect the variety of complex responses of the *in vivo* nervous system is widely recognized. The development of such a pre-screening test, or set of pre-screening tests, is an important goal because of the extensive resources required to examine, by conventional approaches, the large number of new and existing compounds.

Any alternative test methods will have advantages and limitations. Some methods are more appropriate for mechanistic study, whereas others are better suited for screening. The principal advantage of all *in vitro* methods is the possibility of more rigorous control of experimental conditions. Most of the variables that are relevant to the biology of the test system can be controlled in ways which are not possible in animal experiments.

Several limitations characteristic of all alternative test methods give problems in the extrapolation of *in vitro* effects to the *in vivo* situation. Firstly, unless metabolic activators are incorporated in a test system, neurotoxic metabolites of compounds will not be

identified as problem compounds; metabolism of chemicals *in vitro* may be completely different from the *in vivo* situation. Secondly, the absence of a blood-brain barrier, although enabling direct access to the central nervous system, may impair a realistic interpretation of the results. Thirdly, the physico-chemical properties of a compound such as solubility in the test medium, precipitation of essential salts in the growth medium or changes in osmolality of the solutions, may confound the interpretation of results. Fourthly, it is necessary to discriminate between general cytotoxicity and neurotoxicity, e.g., by control studies with tissues or cells of other organs. Finally, the reduced complexity of most alternative test systems may be a disadvantage if the nature of the neurotoxic effect requires a complex integrative system or a long-term exposure of the whole animal to become evident.

In summary, the use of alternative methods to screen for neurotoxicity is likely to be appropriate only in exceptional cases such as compounds of a chemical structure related to known neurotoxicants. *In vitro* methods can be used to obtain important additional information on the mode of action of a neurotoxicant. Consequently, such methods are unlikely to serve as a replacement for common *in vivo* testing, but are more appropriately considered as complementary to whole-animal tests.

SECTION 5 RELEVANCE OF RESULTS FOR MAN

The science of toxicology focuses on the identification of potential toxicological hazards to human health using surrogate animal models in laboratory experiments (Johannsen, 1990). The relevance to man of findings from laboratory animal experiments including toxicological effects in the nervous system (Russell, 1991), is founded on the established biological basis for cross-species extrapolation resulting from the phylogenetic continuity of organisms (Schmidt-Nielsen, 1975; Krasovskij, 1976; Calabrese, 1983).

The evolutionary laws which underlie the uniformity of organisms also account for diversity within and between species in terms of their size, physiology and certain of their metabolic processes (Winneke, 1991). Thus, although many structural, functional and metabolic parameters can be predicted according to mathematical functions of body weight (Krasovskij, 1976; Weiss *et al*, 1977; Calabrese, 1983), interspecies differences, particularly in the toxicokinetics of certain xenobiotics, may be sufficiently large to make cross-species extrapolation and prediction of human toxicological hazard a complex exercise. These somewhat conflicting principles must be taken into account when considering the relevance to man of findings in laboratory species subjected to toxicological tests for behavioural, physiological, biochemical, pathological and developmental effects in the nervous system. The most meaningful cross-species extrapolation of experimental data from test animals to man is ultimately dependent on a knowledge of the molecular mechanism of toxicity as well as any interspecies variation in toxicokinetics and toxicological response.

Behavioural toxicology is a discipline that is still evolving (WHO, 1986). Several investigators support the use of careful clinical observations, or a formalised battery of tests similar to a clinical neurologic examination in man for the initial testing of chemicals in laboratory species (Tilson, 1991). Interpretation of behavioural data may be confounded by systemic toxicity, particularly at high dose levels (Gerber and O'Shaugnessy, 1986). Nevertheless, in animal studies using an appropriate test species and dose rates, clinical evaluation typically can identify potential human neurotoxicants. Examples include several compounds that cause sensory and motor dysfunction in man such as carbon disulphide, acrylamide as well as n-hexane and its metabolites (Spencer and Schaumburg, 1976). The relevance of more complex animal behavioural tests other than clinical observation also is illustrated with chemicals known to induce behavioural deficits in man. For example, the ototoxic effects of toluene in man (Biscaldi *et al*, 1981; Metric and Brenner, 1982) were confirmed by auditory conditioned avoidance tests in rats (Pryor *et al*, 1984) as well as by electrophysiologic studies in rats (Mattsson *et al*, 1990b); also, toluene causes a persistent motor syndrome in rats that resembles to some extent the syndrome (wide-based ataxic gait) in some heavy abusers of toluene-containing products (Pryor, 1991). Another example is the characteristic

"tunnel vision" induced by methyl mercury poisoning in man which was demonstrated in Macaque monkeys using a behavioural test to assess perimetry vision (Merigan *et al*, 1983).

Psychometric-IQ is an endpoint in neurobehavioural studies on adverse environmental factors affecting human cognitive function (Winneke, 1991). Although there is no animal equivalent to IQ-measure, different types of learning and memory tests as developed in experimental comparative psychology are considered as relevant analogues to human cognition and to assess minimal brain dysfunction. These tests may be applied in the context of exposure during either adulthood or development (Elsner, 1991; Winneke, 1991). For example, exposure of adult human beings to tetraethyl lead has produced motor excitability, disorientation and memory impairment as well as disorders of cognition and coordination (Walsh and Tilson, 1984). Behavioural studies in laboratory species, suggesting involvement of the limbic system, produced comparable findings with impairment of memory and learning and increased motor activity and susceptibility to seizure (Walsh and Tilson, 1984). Behavioural studies in man and animals exposed to inorganic mercury have also produced qualitatively comparable findings (Evans *et al*, 1975). Tremor and memory impairment in man related to increased plasma mercury levels were paralleled in rats and other species by tremor and decline in avoidance learning and scheduled operant procedures.

Several investigators have concluded that behavioural studies in laboratory species are relevant in the identification of potential neurodevelopmental toxicants. Kimmel (1988) concluded that there was usually strong qualitative comparability of findings in animals and man for behavioural teratogens. When effects of lead, methyl mercury, polycyclic biphenyls, ethanol, methadone and phenytoin were categorised in terms of behaviour, motor and sensory function, activity and cognitive function, effects in rodents and man were generally similar in terms of occurrence and category of effect (Adams, 1986). Comparisons of the neurodevelopmental effects of alcohol, opioids, cocaine (Hutchings, 1985), and irradiation (Brent, 1980; Jensh, 1986) showed sensitivity of both animals and man to these agents. Contradictory findings have been reported in human and animal studies in the assessment of activity levels (Kimmel, 1988); this reflects the difficulty in designing comparative studies and interpreting data in these types of evaluations.

Neurophysiological tests in laboratory animals are relevant to identify, and in some cases characterise, neurotoxic hazard in man caused by xenobiotic chemicals (Mattsson *et al*, 1989; Winneke, 1991). These techniques, based on phylogenetically common electrophysiological processes, are frequently used in human neurology and can be applied across species (Rebert, 1983; Dyer, 1985). As with other neurofunctional tests, evaluation of results may be complicated when toxicity causes systemic effects such as dietary restriction (Albee *et al*, 1987). Nevertheless, appropriate study design and choice of dose rates can largely prevent these interpretive problems.

Neurophysiological tests have been applied in animals to confirm neurotoxic effects of lead in man. Audiometric deficits associated with increasing blood levels of lead in children and adolescents (Schwartz and Otto, 1988) were paralleled by abnormalities in brainstem auditory evoked potentials in rhesus monkeys (Lilienthal *et al*, 1990). Evidence also was produced for the previously suspected persistence of these effects following cessation of exposure. Effects of lead on the visual system of man and animals have also been described (Grant, 1974). Winneke (1979) and Lilienthal *et al* (1988) later studied flash evoked potentials in rats and rhesus monkeys at low levels of lead exposure. Following studies of pattern-reversal visual evoked potentials in lead exposed children (Otto, 1989), Winneke (1991) concluded from the results of all these studies that there was good cross-species continuity for alteration of visual evoked potential as a measure of deficits in visual information processing due to lead.

2,4-Dichlorophenoxyacetic acid (2,4-D) and dichloromethane are examples of chemicals for which alleged human neurotoxicity following subchronic exposure has been refuted by extensive neurotoxicologic studies in animals. Case reports identified dermal exposure to 2,4-D as a possible cause of human polyneuropathy (Goldstein *et al*. 1959). However, a battery of neurotoxicologic tests to evaluate peripheral nerve function (grip strength, accelerating rod performance, electrophysiology) and structure (light and electron microscopy) in subchronically treated rats did not demonstrate any treatment-related effects (Mattsson *et al*, 1986). Neurotoxicological evaluation of rats exposed to dichloromethane included an observational battery, functional tests, electrophysiology and extensive neuropathology on perfused tissues, but did not show any persistent effects following subchronic inhalation exposure at concentrations up to 2,000 ppm (Mattsson *et al*, 1990a).

The phylogenetic continuity of species has been recognised by the medical community as the basis for the use of animal models to study biochemical and pathomorphological response in all types of disease, including those resulting from over-exposure to hazardous xenobiotic chemicals (RCP, 1990). In neurotoxicology, animal models were used extensively to study the spatiotemporal pattern of lesions resulting from tri-o-cresyl phosphate (TOCP) poisoning (Cavanagh, 1963) following reports of this peripheral neuropathy in man (Aring, 1942). Since the initial reproduction of the TOCP syndrome in laboratory species, many other animal models of human neurological syndromes have been readily produced to study the development of pathological changes (Spencer and Schaumburg, 1976). Examples of chemicals that cause central or peripheral nervous system lesions and which have been studied in animal models are listed in Appendix D. Study of the neuropathological response in animal models with such diverse chemicals as organophosphates (Cavanagh, 1954, 1964), hexachlorophene (Towfighi and Gonatas, 1976), lead (Pentschew and Garro, 1966) and lovastatin (Berry *et al*, 1988) indicate that susceptibility may vary, depending on the age, species and strain of animal. Nevertheless, the important principle remains that the

identification of neuropathological changes in experimental animals raises the possibility of a potential hazard to human health. The relevance of animal models in the definition of a confirmed human neurotoxicant is also emphasised by Allen (1980) and Spencer and Schaumburg (1985). Suspected human neurotoxicants should not finally be classified as known human neurotoxicants until the syndrome has been reproduced in a laboratory species.

SECTION 6 PRACTICAL TESTING STRATEGY

6.1. ADEQUACY OF DATA

Studies that investigate the potential effects of chemicals on the nervous system should provide adequate data for risk assessment in order to protect human health in the work place and in the environment. These data should include the following:

- **nature of neurotoxic effect** - determine if the chemical affects the central, peripheral or autonomic nervous system and if the material causes morphological effects and/or functional changes;

- **dose response** - the dose response and NOEL should be established;

- **transient versus persistent effects** - the time course and persistence of neurological effects should be determined;

- **direct/indirect effect** - establish whether the neurotoxic effects are direct or secondary to some other toxic effect.

Additional information may, in some cases, be necessary for adequate hazard assessment. Examples include:

- **species specificity** - susceptibility of a second animal species may be investigated. Activity in a second species suggests that the chemical is more likely to be active in man;

- **toxicokinetics** - investigation of toxicokinetics of chemicals would further the understanding of toxic effects in animals and may provide an indication of the relevance of the animal data to man;

- **mechanism of action** - the mechanism for a neurotoxic effect in animals may provide a better understanding of the potential for neurotoxicity in man.

The adequacy of available toxicity data from animal studies as well as any information from human exposure should be evaluated in relation to the need for hazard assessement. The depth of an assessment of the potential risk to man depends on the use of a chemical and the risk of human exposure. Inadequate data may need to be supplemented by additional studies in order to estimate the neurotoxic potential of a chemical and allow risk assessment.

Clinical observations and histological examination of nervous tissue are central to the identification of potential neurotoxicity and are performed in most standard toxicity studies. Enhancement of these studies by additional relevant parameters and careful evaluation of data from all of these studies would broaden the data basis for identification of a potential neurotoxic effect. The chance for detecting a potential neurotoxic effect is increased by not relying on a single species and route of administration. The evaluation of relevant data in a special report could eliminate the need for separate screening tests to identify potential neurotoxicity. Such an approach can be applied to new as well as existing chemicals and would reduce the amount of testing as well as expedite the assessment of the neurotoxic risk of chemicals.

6.2. TEST STRATEGY

A test strategy for the evaluation of the neurotoxic potential of chemicals should not be rigid, but should be determined with a case-by-case approach and depend on such factors as the structure and physical form of the chemical, systemic toxicity, and nature of any neurotoxic response. General guidance for a test strategy is schematically presented below (cf. page 32).

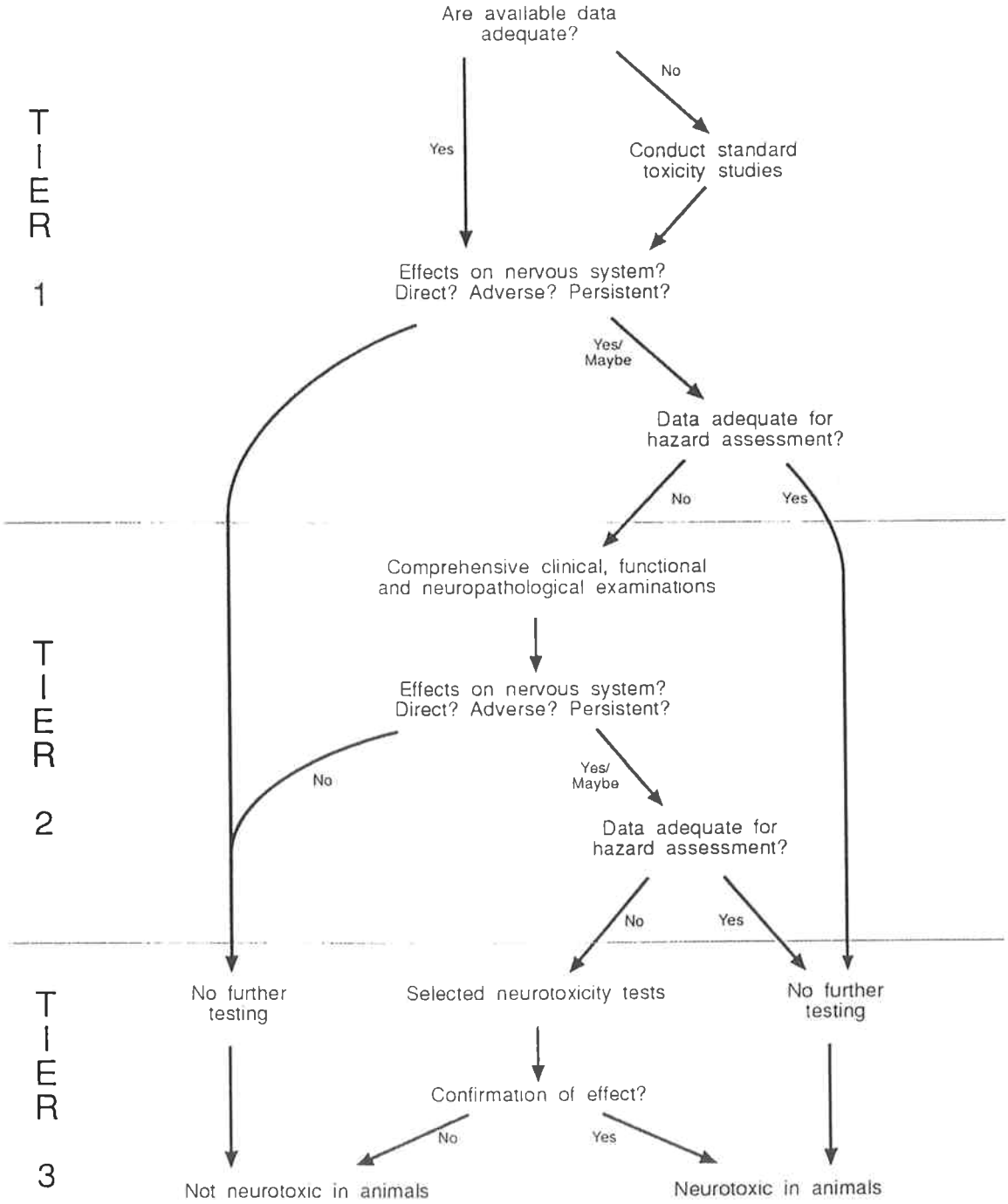
6.2.1. Tier 1 - Screening Tests

As discussed in Section 4.1. standard toxicity studies normally detect effects in the nervous system as well as effects in other body systems such as the digestive, urinary or cardiovascular systems. Standard toxicity studies are reliable Tier 1 "screening" tests for the detection of potential neurotoxicity and often are adequate for hazard assessment. Standard toxicity studies are appropriate for neurotoxicity screening when the available data do not suggest a potential for neurotoxicity.

Study design and requirements. Standard toxicity studies are conducted at relatively high dose levels with different durations and routes of administration and examine several species of animal. The number of animals required for standard studies are adequate for Tier 1 assessment of potential neurotoxic effects.

Carefully conducted and well documented clinical observations are important components of any toxicological evaluation and are essential for the assessment of potential neurotoxicity. Clinical signs should be recorded as they are observed and should include the time of onset, degree, and duration of effects. Observations should involve handling the animal and include observations inside and outside of the cage.

TEST STRATEGY FOR NEUROTOXICITY HAZARD ASSESSMENT



Parameters should include changes in skin, fur, eyes, mucous membranes, occurrence of secretions, changes in spontaneous behaviour pattern, activity, gait, muscular tone, sensory activity (e.g., response to visual, tactile or auditory stimulus), and autonomic activity (e.g., lacrimation, pupil size, piloerection, skin temperature, and unusual respiratory patterns).

A quantified measurement of grip strength or motor activity is not appropriate as a component of standard toxicity studies because of the confounding influence of systemic toxicity. Such investigations may be useful in more detailed studies on potential neurotoxicity (Tier 2) if conducted at dose levels that do not produce systemic toxicity.

Routine preservation of the brain, spinal cord, peripheral nerves and skeletal muscle in fixative should be in accordance with standard toxicity test guidelines. The brain should be included in the list of tissues to be weighed (ECETOC, 1991). Histopathological examination should include the brain and a peripheral nerve; the spinal cord and skeletal muscle should be examined if other findings suggest a possible treatment related effect. Perfusion fixation of nervous tissue is not necessary for Tier 1 studies.

Evaluation. When there is no evidence of nervous system effects either in laboratory animals from standard toxicity tests or from experience of human exposure, and when the chemical structure of the substance suggests no concern for potential neurotoxicity, then the substance can be regarded as not neurotoxic. In this case, no further testing is warranted until any new cause of concern for the nervous system arises.

When standard toxicity studies, perhaps in conjunction with other data, provide sufficient evidence of direct, adverse, persistent effects on the nervous system, the chemical can be considered a probable human neurotoxicant. If the weight of evidence is sufficient and the data are adequate for hazard assessment, then no further testing is warranted.

When there is equivocal evidence of direct, adverse, persistent effects on the nervous system in standard toxicity studies and/or if there is a plausible structure relationship with known neurotoxicants, the chemical could be considered a possible human neurotoxicant. Tier 2 neurotoxicity studies should then be considered.

6.2.2. Tier 2 - Neurotoxicity Tests

Chemicals of concern are those that have structure/activity relationships to known neurotoxicants or those chemicals for which there is an indication of neurotoxic potential from standard toxicity studies or human exposure. These chemicals should be tested further if the available data are inadequate for risk assessment.

The goal of Tier 2 tests is to thoroughly evaluate the nervous system with broad, exploratory tests and to provide adequate data for risk assessment. Because of the

multiplicity of possible effects in the nervous system. there is no single test method that can ensure the detection and identification of every possible change. Therefore, Tier 2 tests should evaluate major structures and functions of the nervous system. In certain circumstances Tier 2 test methods may be employed in conjunction with standard toxicity studies.

Study Design and Requirements. A subchronic study that incorporates specialised evaluations of the nervous system is recommended for Tier 2 testing since the study design allows for possible accumulation of a chemical within the body or the expression of delayed neurotoxic effects. If toxicokinetic parameters indicate rapid elimination, shorter studies may be appropriate.

The highest dose level for Tier 2 studies should challenge the nervous system, but should not be so high that there are complications from systemic toxicity.

Assessment of clinical signs should be as detailed as for the Tier 1 tests and may be supplemented by functional tests covering sensory, motor and autonomic nervous functions including testing of reflexes. These investigations normally include semi-quantitative estimation of animal locomotion. Measurement of motor activity with automated devices may provide additional but non-specific indications for potential neurotoxic effects.

Neuropathological evaluations should be performed on high-dose and control animals first and intermediate-dose animals only if necessary. Histological examination of central and peripheral nervous system should include as a minimum the following tissues: brain (several sections), spinal cord (cervical and lumbar enlargements, spinal roots, dorsal root ganglia), and peripheral nerves (e.g., sciatic and tibial nerves). Routine histopathology should include examinations of brain structures that are related to specific types of behaviour (e.g., the hippocampus, the hypothalamus, the cerebellum etc.). Additional tissues, special fixation (perfusion) and staining techniques may be necessary depending on preliminary findings. Neuropathological investigation may be supplemented by electron microscopic, morphometric or histochemical examinations of selected specimens to further clarify the diagnosis.

These neurotoxicological investigations can be incorporated into standard toxicity studies in order to provide evaluation of nervous system effects in relation to any other toxic effects of the chemical and minimise the use of additional animals. This also will help to establish whether the neurotoxic effect is direct or secondary to toxic effects in other organs.

Evaluation. Tier 2 tests will provide adequate data for risk assessment in most cases. When there is no evidence of a neurological effect in Tier 2 studies then the substance can be regarded as not neurotoxic. In this case no further testing is warranted unless new evidence suggests concern for a potential neurological effect.

When direct, adverse, persistent effects on the nervous system are detected or confirmed with Tier 2 tests, the chemical may be considered a 'probable human neurotoxicant'. When the weight of evidence is sufficient and the data are adequate for risk assessment, no further testing is warranted.

Further specific neurotoxicity tests may be warranted in a Tier 3 characterisation phase if the effects on the nervous system are unclear or inconsistent.

6.2.3. Tier 3 - Characterisation of Neurotoxicity

A decision to conduct Tier 3 neurotoxicity studies depends upon factors such as the nature of the neurotoxicity, the potential of the chemical to accumulate in biological systems, the intended use of the chemical and the potential for human exposure. Neurotoxicity data from Tier 1 and Tier 2 studies should provide a majority of the information that is necessary for risk assessment. Tier 3 neurotoxicity tests may be appropriate for the advanced characterisation of known neurotoxicants in selected instances when Tier 1 and Tier 2 evaluations are inadequate for risk assessment. Investigation of the mechanism of action or toxicokinetics of the chemical may also form part of a Tier 3 evaluation.

Specific Neurotoxicity Methods. A case-by-case approach is necessary to determine the most appropriate methods for Tier 3 studies. The data from Tier 1 and Tier 2 studies should provide a basis for generation of a refined hypothesis for Tier 3 studies and guide selection of the most appropriate methodology. The following are categories of possible Tier 3 studies:

- cognitive function or other specialised behavioural methods;
- electrophysiological methods;
- neurochemical methods;
- specialised neuropathological techniques.

More detailed information is provided in Section 4 and Appendix C.

Evaluation. Specific hypotheses resulting from findings in Tier 1 or Tier 2 studies may not be confirmed in Tier 3 studies. If there is no additional evidence of neurotoxicity and the hypothesis is rejected, then the substance can be regarded as not neurotoxic. In this case no further testing is warranted unless new evidence suggests concern for a potential neurological effect.

When direct, adverse, persistent effects on the nervous system are confirmed with Tier 3 tests, the chemical should be considered 'a probable human neurotoxicant'. When the weight of evidence is sufficient and the data are adequate for risk assessment, no further testing is warranted.

6.3. SPECIFIC COMMENTS ON ACUTE NEUROTOXICITY TESTS

Acute tests which typically are conducted at lethal or near-lethal dose levels are considered to be of limited value for the assessment of neurotoxicity. Substances which are not directly toxic to the nervous system can be associated with signs such as convulsions, tremors or ataxia at systemically toxic or lethal dose levels. Subacute or subchronic studies would provide more interpretable data on the nervous system because of the decreased severity of systemic toxicity as compared to acute (lethal or near-lethal) studies. We are not aware of any chemical that damages the nervous system after a single exposure that does not also have neurotoxic effects after repeated exposure although the neurotoxic effects are not necessarily the same (Yoshimura *et al*, 1992). Thus, emphasis on subacute or subchronic studies for the evaluation of the nervous system will provide adequate data for the protection of human health.

6.4. SPECIFIC COMMENTS ON DEVELOPMENTAL NEUROTOXICITY

Standard reproduction studies can detect disturbances in nervous system development. Thus, standard studies should incorporate an evaluation of physical and functional landmarks which assess sensory and motor function. These enhanced studies could then be considered as true screening tests for developmental neurotoxicants and in most cases would provide adequate data for hazard assessment.

When data are not adequate for hazard assessment, enhanced reproduction studies may be followed by more specific testing to further investigate and characterise the functional deficit. A case-by-case approach is necessary to determine the most appropriate methods for these special studies. Examples of possible methods are listed in Section 6.2.3. above and in Table C-9.

SECTION 7 CONCLUSIONS AND RECOMMENDATIONS

Potential effects of chemicals on the nervous system are difficult to assess because of the complexity of the nervous system. All chemicals are toxic at some dose level and will induce some behavioural change at these dose levels. Thus, essentially all chemicals could be classified as "neurotoxic", if behavioral changes or neurological signs are accepted as unerring indicators for neurotoxicity. Therefore, detection of compounds that cause direct, persistent, adverse effects on the nervous system should be given the most critical attention in order to protect human health in the workplace and the environment.

Evaluation of the neurotoxic potential of a chemical should include descriptions of functional and morphological effects as well as the determination of the dose response, time course, reversibility of effects and the NOEL. Differentiation between direct and indirect neurotoxic effect is critical for scientific and regulatory considerations. Also, investigation of species specificity, toxicokinetics and the mechanism of a neurotoxic effect may provide useful information.

A tiered testing strategy is recommended for the evaluation of the neurotoxic potential of chemicals. This approach should be flexible and guided by such factors as the structure and physical form of the chemical, the potential human exposure and the nature of any neurotoxic effect.

Evaluations of the nervous system in the context of standard toxicity studies are Tier 1 screening tests for the detection of potential neurotoxicity. Standard toxicity studies are conducted at relatively high dose levels with different durations and routes of administration and examine several species of animal. There are numerous examples in the scientific literature that demonstrate the ability of standard toxicity studies to detect a variety of neurotoxic effects.

Validated methods for the evaluation of the nervous system can, where necessary, become part of standard toxicity studies. Some existing study guidelines (e.g., OECD Guideline 407, Repeated Dose Oral Toxicity - Rodent) should be strengthened by the inclusion of additional clinical and pathological examinations related to the nervous system (ECETOC, 1991). Standard reproduction studies could be extended to include physical and functional landmarks covering sensory and motor function. Additional emphasis on the evaluation of the nervous system within the context of standard toxicity studies would eliminate the need for separate guidelines for neurotoxicity screening. Data relevant for identification of potential neurotoxicity from available standard toxicity studies could be summarised and evaluated in a separate report.

Tier 2 studies should be performed with chemicals for which there is an indication of a neurotoxic potential and the available data are inadequate for risk assessment. The goal of Tier 2 studies is to evaluate thoroughly the nervous system with broad, exploratory tests that will provide adequate data

for risk assessment. Tier 2 studies should evaluate the major functions and structures of the nervous system by comprehensive clinical examinations and neuropathological assessment. These studies may be conducted in conjunction with standard toxicity studies such as subchronic studies so that any potential neurotoxicity can be interpreted in the context of other systemic toxicity. Tier 2 tests will be adequate for risk assessment in most cases and only rarely would further testing be required to provide additional information essential for the protection of human health.

More specific Tier 3 neurotoxicity tests may be conducted for advanced characterisation of known neurotoxicants with carefully selected techniques such as neurophysiology, neurochemistry, observation of behaviour or neuropathology. Investigation of the potential of the chemical to induce developmental neurotoxicity also may be considered at this stage. A decision as to the need for Tier 3 studies depends on numerous factors such as the type of neurotoxic effect, the potential of the chemical to accumulate, the use of the chemical, and the potential human exposure. Advanced characterisation may be appropriate if Tier 1 and Tier 2 studies are insufficient or inadequate for risk assessment.

APPENDICES

APPENDIX A GLOSSARY OF TERMS

Acetylcholinesterase (AchE): Enzyme released into the synaptic space to inactivate the neurotransmitter acetylcholine. Its inhibition accounts for the acute form of organophosphorus intoxication.

Aging (of NTE-neurotoxic esterase): The process by which inhibition of NTE by an organophosphorus compound increases due to covalent binding to the esterase.

Astrocyte: A multifunctional Central Nervous System (CNS) glial cell with numerous processes abutting on capillaries and neurons.

Autonomic Nervous System: The division of the nervous system which principally controls the non-cognitive, normal physiological function of organs including skin, cardiovascular and respiratory systems, as well as the alimentary and urogenital tracts.

Axons: The longest process of a neuron (which may be >1 meter in man) which conducts either motor or sensory impulses in the CNS and PNS. Axons may be myelinated or non-myelinated.

Axonopathy: A degenerative condition of axons which may potentially result from exposure to a neurotoxic chemical.

Blood-brain-barrier: Permeability barrier consisting of vascular endothelium, basement membranes, neuroglial membranes and glial perivascular end-feet by which many, particularly high-molecular weight substances are excluded from the brain. This system preserves homeostasis in the nervous system, facilitating the entry of necessary metabolites, but blocking entry or facilitating removal of unnecessary metabolites or toxic substances.

Central Nervous System (CNS): The part of the nervous system contained within the cranium and the vertebral column, i.e., the brain and spinal cord.

Central-Peripheral Distal Neuropathy: A neuropathy affecting the distal segments of axons in the central and peripheral nervous system.

Chromatolysis: An adaptive or degenerative response of neurons often resulting from axonal damage. Typically, the neuronal nucleus becomes eccentrically displaced and the cytoplasmic Nissl substance (rough endoplasmic reticulum) is dispersed or peripherally distributed in the perikaryon, resulting in a pale eosinophilic 'ground glass' appearance of the cytoplasm by light microscopy.

Dark (basophilic) Neurons: Shrunken strongly basophilic neurons with a spiky electron-dense appearance by transmission electron microscopy. Dark neurons occur as a post-mortem artefact or in certain acute neurodegenerative processes.

Degeneration: Metabolic and morphological cellular changes which, if not reversed, lead to necrosis or cell death.

Delayed Neurotoxicity: The process by which evidence of neurotoxicity is not clinically evident until sometime after exposure and typified by OPIDN (organophosphorus induced delayed neuropathy).

Dendrites: The multiple neuronal processes on which axons (of other neurons) synapse.

Distal Neuropathy: A degenerative condition initially affecting the segments (parts) of axons most distal from the neuronal perikaryon.

Dysgenesis: The abnormal development of an organ, such as the nervous system, and potentially caused by *in utero* or early post-natal exposure to a neurotoxic substance. In the mature nervous system dysgenesis may be evidenced microscopically by ectopic neurons, reduced numbers of neurons and neurites and abnormal microscopic anatomy.

EEG: Electroencephalogram. Electrophysiological measure of the dynamic process of integrated electrical activity of the central nervous system.

Encephalopathy: A degenerative condition affecting the brain.

Evoked potential: Electrical signal generated by the nervous system in response to a stimulus. Stimulus may be auditory (brainstem auditory evoked potentials, BAEPs), visual (visual evoked potential, VEP) or somatosensory (somatosensory evoked potential, SSEP).

Ganglion: A focal collection of neurons, either outside the CNS and surrounded by fibrous connective tissue (e.g., the dorsal root ganglia of the spinal cord comprising groups of sensory neurons), or within the brain (e.g., basal ganglia).

Germinal Layer: A layer of germinal cells in the foetal nervous system from which precursor neurons migrate to their permanent location in the mature nervous system, e.g., the external molecular layer of the immature rat cerebellum. Exposure of the developing CNS to neurotoxic compounds can potentially disrupt neuronal migration.

GFAP (Glial fibrillary acidic protein): The intermediate-filament cytoskeletal protein specific to astrocytes in the central nervous system.

Gliopathy: A pathological process affecting glial cells and potentially induced by neurotoxic chemicals such as nicotinamide.

Hazard: According to the EEC definition, hazard is a potential toxic effect which may be caused by a chemical following exposure.

Hazard Assessment: According to the EEC definition, the hazard assessment means to assess the potential for a substance to cause adverse effects on environmental species and/or man. Therefore, the hazard assessment needs information on environmental exposure (environmental compartments of concern, quantities) and effect data (with reference to the environmental compartment of concern) and is normally expressed by a comparison of the (predicted) environmental concentration with the (predicted) no effect concentration for concerned species or ecosystems.

Hazard Identification: The hazard identification aims at a first stage to identify a substance as a substance of concern.

Hippocampus: Essential component of the brain circuitry that comprises the limbic system. It processes information derived from the association cortices and in turn influences cortical and subcortical areas that modulate complex behavioural processes.

Ischemia: Interruption or marked reduction of the blood flow.

Ischemic Cell Change: The term used in neuropathology to describe morphological changes in neurons caused by anoxia, ischemia and related toxicological or metabolic processes.

Lesion: A morphological change associated with a pathological condition.

Leukoencephalopathy: A degenerative condition particularly affecting white matter of the brain.

Malacia: A term used in neuropathy to describe vacuolation in the CNS observed by light microscopy. Malacia may vary in severity and be focal, diffuse or laminar in distribution. Cerebrocortical laminar malacia may occur in neurotoxic conditions.

Mid-air righting reflex: Term used to describe the process by which experimental animals, usually rats, assume normal postural orientation when falling. The test animals are dropped in a supine position from about 60 cm height onto a padded surface and the landing position is observed. The reflex involves rotation of head, forelimbs, and hind-limbs. The righting reflex can be partially or totally abolished under certain neurotoxic conditions.

Motor Nerves: see motor neurons.

Motor Neurons: Neurons concerned with innervation of skeletal muscle. The cell body of lower motor neurons are located in the ventral horn grey matter of the spinal cord and their axons directly innervate skeletal muscle. Lower motor neurons are in turn innervated by upper motor neurons with cell bodies located in the brain.

Myelin: A lipoproteinaceous insulating sheath surrounding axons essential for achieving high speed conduction.

Myelinopathy: A pathological process primarily affecting myelin, such as that caused by the neurotoxin hexachlorophene, in which myelin sheaths of the central and peripheral nervous system form large intramyelin vacuoles. Secondary axonal degeneration may result.

Myoneural junction: see neuromuscular junction.

Myopathy: A degenerative process affecting muscle.

Necrosis: Cell death.

Nerve growth factor (NGF): Protein which is thought to be a trophic substance critical for the development and maintenance of sympathetic neurons.

Neuronopathy: A degenerative process primarily affecting neurons. Morphological changes may be first observed in the neuronal perikaryon or the proximal or distal axon.

Neurotoxic esterase (NTE): The membrane bound axonal enzyme which is inhibited in the organophosphorous-induced delayed neuropathy (OPIDN).

Neurotoxicity: An adverse change in the structure or function of the nervous system that results from exposure to a chemical substance.

Nissl substance: The light microscopic term to describe the basophilic concentrations throughout the neuronal perikaryon and which are confirmed as rough endoplasmic reticulum by transmission electron microscopy.

NCV: Nerve conduction velocity. The speed at which a nerve conducts an action potential.

Oligodendrocyte: The glial cell which forms myelin sheaths of axons in the central nervous system.

Operant behaviour: Associative learned behaviour which involves associating a response with a stimulus (reward or punishment).

Peripheral Nervous System: That part of the nervous system outside the brain and spinal cord, i.e., peripheral nerves and ganglia, including those of autonomic function.

Pinna detachment: Unfolding of the external ear (the projecting part of the ear lying outside of the head); in Sprague-Dawley rats usually observed from day 2 after birth onwards.

Pivoting: Pivoting is a circular locomotory movement of neonatal rat pups resulting from the fact, that in up to 4-to 5-day old rats the forelimb function is better developed than hind-limb function.

Proximal neuropathy: A degenerative process primarily affecting the proximal segment of axons.

Pyknosis: The process by which the nuclear chromatin of cells undergoing necrosis condenses to form a dense basophilic body. Nuclear pyknosis is typically seen in neurons which have undergone rapid degeneration and necrosis associated with acute ischemia or excitotoxin-induced neuronal degeneration.

Radiculoneuropathy: A neuropathy affecting the roots of nerves of the spinal cord.

Reference memory: Information stored over the long term that is relatively resistant to interference.

Risk: According to the EEC definition, risk is the measured or estimated probability of a toxic response following exposure to a toxic chemical. It may be expressed semi-quantitatively, for example as high, medium or low, or quantitatively in absolute terms as the probability of occurrence of an effect.

Risk Assessment: The term risk assessment is often confused with the term hazard assessment; it is frequently used as a comprehensive term to cover any kind of adverse effect evaluation of substances. As a risk usually is expressed as the probability of the occurrence of an adverse effect, the term "risk assessment" should not be used if no probabilities are calculated. The risk assessment means to estimate the probability that a substance causes adverse effects as a result of the (at a given concentration) presence of that substance in the environment.

Safety factor: Synonymous with uncertainty factor (UF) according to the US-EPA, is the factor applied to calculate an acceptable daily intake (ADI) and which allows for uncertainties in extrapolation to man from experimental studies in animals. The magnitude of an overall safety factor will increase with allowance for factors such as inter- and intraspecies variation, extrapolation from studies of less than chronic duration, availability of experimental data from only one laboratory species and use of a LOAEL (lowest observable adverse effect level) rather than a NOAEL (no observable adverse effect level).

Sensory Neurons: Neurons which lead to the CNS and transmit information from the receptors about changes in the environment or the body.

Silent Lesion: A lesion which is not immediately evident but may appear later in life or at a later stage of a disease process.

Surface righting reflex: A reflex response in laboratory animal species. The test animals are placed in a supine position on a horizontal surface and the time required to resume normal posture is measured.

Swimming ontogeny: A developed ability assessed in laboratory animals to assess effects of potential developmental toxicants. The development of swimming ability in growing rats can be scored with respect to body position and limb usage.

Synaptosome: Subcellular membrane structure; entire synaptic terminal produced by gentle grinding of neurons in an isotonic medium; can be isolated by differential centrifugation and releases synaptic vesicles when diluted into water.

Threshold: The threshold dose of a toxic chemical is the dose level below which a toxic response for a particular toxicologic end point is not detected. Thus, the no effect level is the highest experimental dose below the threshold dose.

Wallerian Degeneration: Originally described by Waller, the degenerative changes observed by light microscopy in the distal segment of axons severed from their parent neurons. Morphologically similar axonal degeneration is observed in many neuropathies and is loosely referred to as Wallerian degeneration but should strictly be termed Wallerian-like degeneration.

Working memory: Recall of recent events of transient importance that is vulnerable to interference.

APPENDIX B-1 NEUROTOXICITY TESTING GUIDELINES

	OECD draft guidelines March 1990	EPA/FIFRA guidelines March 1991	Japanese MAFF guidelines 1985
Indication	- neurological signs - neuropathology - structural relationship to known neurotoxicants	routine investigation for chemicals	NO SPECIFIC GUIDELINES FOR GENERAL NEUROTOXICITY TESTING
Species, Age and Sex	rats, young adults, males and females	rats, at least 42d, males and females	
Group Size	10/group (5♂+5♀) 6 high-dose + controls for neuropathology	10/sex/group 5/sex for neuropathology	
Control Groups	vehicle control	as for OECD	
Postitive Control Data		- hind limb weakness - tremor - automatic signs - CNS and PNS pathology	
Dose Levels	3 doses Acute exposure: Limit test at 2g/kg acceptable Repeated exposure: high-dose: no severe toxic effects. low-dose: NOAEL. daily administration (5d/wk acceptable), limit test at 1g/kg.	3 doses Acute studies: High-dose: highest nonlethal dose (≤2g/kg). Subchronic studies: high-dose: clear toxic effects (≤1g/kg). low-dose: minimal or no effects.	
Route	Consider: - human exposure - kinetics	Consider: - human exposure - kinetics - practicability	
Observation Procedures	- blind - structured procedure - on standard arena - description and ranking	- blind - standard procedure - in home cage, open field, manipulations - description and ranking	

	OECD draft guidelines March 1990	EPA/FIFRA guidelines March 1991	Japanese MAFF guidelines 1985
Frequencies	- pretest - frequency must allow detection of effects	Acute studies: pretest, peak effect, test day 7 and 14. Subchronic studies: pretest, week 4, 8 and 13. Chronic studies: pretest and every 3 months.	
Observations	- convulsions, tremor - hyperactivity - aggression - stereotypes - autonomic signs - abnormal posture or gait - activity level - abnormal respiration - vocalization	as for OECD	
Measurements	- sensory functions - grip strength - motor activity	as for OECD & additionally: - body weight - landing foot splay	
Neuropathology	Acute exposure: - gross necropsy of brain - gross pathology - brains trimmed Repeated exposure: - in situ fixation - paraffin/plastic embedding - H&E or comparable stain - special stains if necessary	Acute exposure: as for repeated exposure of OECD Repeated exposure: as for OECD	

	OECD draft guidelines March 1990	EPA/FIFRA guidelines March 1991	Japanese MAFF guidelines 1985
Tissues/Sections	- brain (5 levels) - spinal cord (2 levels) - dorsal root ganglion - dorsal/ventral roots - prox. sciatic and prox. tibial nerves - skeletal muscle - other sites if necessary	cover all major regions of nervous system	
Report	tabulate results (incidence and severity) - evaluate results - correlate neuro-pathology/clinical signs/FOB	as for OECD & additionally describe: - methods/equipment - standardisation procedures - scoring criteria	
Interpretation	In case of neuro-toxicity and further data to allow quanti-tative analysis of effects	not specified	

APPENDIX B-2 DELAYED NEUROTOXICITY OF ORGANOPHOSPHOROUS SUBSTANCES FOLLOWING ACUTE EXPOSURE

	OECD guideline no. 418, April 4 1984	EPA/FIFRA guidelines March 1991	Japanese MHW guidelines 1985
Indication	organophosphorous substances	as for OECD	as for OECD
Species, Age and Sex	hens, 8-14 months	as for OECD	as for OECD
Group Size	≥6 hens at end of observation period	as for OECD	as for OECD
Control Groups	≥6 vehicle controls	as for OECD & in addition: 3 hens for NTE	as for OECD
Positive Control	≥2 hens - TOCP or - Leptophos	≥6 hens plus ≥3 hens for NTE historic data sufficient	≥4 hens - TOCP etc.
Dose Levels	≤LD_{50} but (max 5g/kg) - repeat if negative or equivocal results - atropine to protect animals (including controls)	≤LD_{50} or ALD (max 2g/kg) - repetition not required as for OECD	≥LD_{50} (max 5g/kg) as for OECD as for OECD
Route	oral	as for OECD	as for OECD
Observation procedures	record onset, degree, duration	as for OECD	as for OECD
Frequencies	once daily for at least 21d	as for OECD	as for OECD
Observations	- behavioural abnormalities - ataxia, paralysis - 2 times/wk forced activity (eg. ladder climbing)	as for OECD	as for OECD
Measurements	body weight (weekly)	as for OECD	as for OECD
NTE, AChE	not required	3 hens at 48h after dosing (other time if detection optimised) duplicate measures - brain - spinal cord	as for OECD

	OECD guideline no. 418, April 4 1984	EPA/FIFRA guidelines March 1991	Japanese MHW guidelines 1985
Neuropathology	gross necropsy recommended - in situ fixation - myelin and axon specific stains	as for OECD	as for OECD & additionally: - H&E stain
Tissues/Sections	- medulla oblongata - spinal cord (3 levels) - prox. tibial nerve	as for OECD	as for OECD & additionally: - cerebellum - cortex
Report	tabulate results - effects - lesions - body weight	as for OECD & additionally: - biochemical data	not specified

APPENDIX B-3 DELAYED NEUROTOXICITY OF ORGANOPHOSPHOROUS SUBSTANCES FOLLOWING SUBCHRONIC EXPOSURE

	OECD guideline no. 419, April 4 1984	EPA/FIFRA guidelines March 1991	Japanese MHW/MAFF guidelines 1985
Indication	- delayed neurotoxicity following acute exposure - inhibition and ageing of NTE	as for OECD	as for OECD
Species, Age and Sex	hens, 8-14 months	as for OECD	as for OECD
Group Size	10 hens/group	≥6 hens plus ≥3 hens for NTE	as for OECD
Control Groups	vehicle controls	as for OECD & additionally: 3 hens for NTE	as for OECD
Positive Control	not required	≥6 hens plus ≥3 hens for NTE historic data sufficient	10 hens - TOCP etc.
Dose Levels	3 doses high-dose: toxic effect but no fatalities low-dose: NOEL	3 doses high-dose: maximum tolerated dose (max. 1g/kg) low-dose: LOEL or NOEL	3 doses high-dose: slight toxic effect (ChE depression)
	daily administration for 90d (5d/wk acceptable)	daily administration for 28d	as for OECD as for OECD
Route	oral	as for OECD	as for OECD
Observation procedures	record onset, degree, duration	as for OECD	as for OECD
Frequencies	once daily	as for OECD	as for OECD & additionally: - 7d recovery period
Observations	- behavioural abnormalities - ataxia, paralysis - once/wk forced activity (eg. ladder climbing)	as for OECD twice/wk forced activity	as for OECD
Measurements	body weight (weekly)	as for OECD	as for OECD

	OECD guideline no. 418, April 4 1984	EPA/FIFRA guidelines March 1991	Japanese MHW guidelines 1985
NTE, AChE	not required	3 hens at 48h after dosing (other time if detection optimised) duplicate measures - brain - spinal cord	as for OECD
Neuropathology	gross necropsy recommended in presence of clinical signs - in situ fixation - myelin and axon specific stains	as for OECD	as for OECD but no gross necropsies additionally: - H&E stain
Tissues/Sections	- medulla oblongata - spinal cord (3 levels) - prox. tibial nerve - sciatic nerve	as for OECD	as for OECD & additionally: - cerebellum - cortex - peripheral nerves (3 levels) - myoneural junction
Report	tabulate results (incidence and severity) - effects - lesions - body weight	as for OECD & additionally: - biochemical data	not specified
Interpretation	in conjunction with results of acute study		

APPENDIX B-4 DEVELOPMENTAL NEUROTOXICITY SCREEN

	OECD guideline no. 416, 1983 (2 generation reproduction)	EPA/FIFRA guidelines March 1991	Japanese MHW/MAFF guidelines 1985
Indication	routine investigation	- structural relationship to known neurotoxicant - neurotoxic chem. - neuroactive chem. - chemicals affecting hormones associated with brain development	routine investigation for pharmaceuticals
Species	rats	as for OECD	as for OECD
Group Size	20 litters, culled to 4♂+4♀ on PN4	20 litters, culled to 4♂+4♀ on PN4 1♂+1♀ for each of: - motor activity - audit. startle - learning/memory 10♂+10♀ for neuropathology PN11/62	Segm. II: 30 dams of which 20 autopsied at term, 10 dams deliver & each litter culled to 4♂ and 4♀ on PN4 Segm. III: 20 litters each culled to 4♂ and 4♀ on PN4
Control Groups	vehicle controls	as for OECD	as for OECD
Positive Control	not required	to demonstrate sensitivity/ competence	not required
Dose Levels	3 doses high-dose: induce toxicity in dams low-dose: NOEL limit test at 1g/kg acceptable	3 doses high-dose: - not inducing *in utero* malformations or death bodyweight gain reduced by ≤20%	3 doses high-dose: toxicity in dams
Dosing Period	continuous	G6-PN10	Segm. II: G7-G17 Segm. III: G17-PN21

	OECD guideline no. 416, 1983 (2 generation reproduction)	EPA/FIFRA guidelines March 1991	Japanese MHW guidelines 1985
Route	oral	as for OECD	as for OECD
Observation Dams	daily: - signs of toxicity - behavioural changes - difficult or prolonged parturition - litter size, still/live births weekly: - body weight - food consumption	daily: - autonomic signs - convulsions/tremor - abnormal posture/ gait/movements - abnormal behaviour/ appearance periodically: - body weight	daily: - behavioural changes - signs of toxicity - difficult or prolonged parturition
Offspring	- Physical/ behavioural abnormalities landmarks: - bodyweight PN1, 4, PN7, and weekly	whenever removed from cages - observations as for dams landmarks: - bodyweight PN1, 4, PN11, 17, 21 - vaginal opening - motor activity PN13, 17, 21, 60 - audit. startle PN22, 60 - learning/memory PN21-24, 60	- observations - physical/ behavioural abnormalities landmarks: - growth and development (morphological/ functional/behavioural examination)
Pathology	- gross necropsy - macroscopic/structural abnormalities	not required	as for OECD
Tissues/Sections	- organs of reproductive system - target organs	not required	target organs

	OECD guideline no. 416, 1983 (2 generation reproduction)	EPA/FIFRA guidelines March 1991	Japanese MHW guidelines 1985
Neuropathology PN11:	not required	10♂+10♀: brain weight 6♂+6♀: brains, immersion fixed - paraffin/plastic embedding - H&E stain - examine major brain regions	not required
PN62:		10♂+10♀: brain weight 6♂+6♀: in situ fixation - paraffin (CNS) or plastic (PNS) embedding - H&E stains - examine stepwise - special stains if necessary	
Report	- tabulate results (incidence and severity) - evaluate results	as for OECD & additionally: - correlate neurological/pathological behaviour	as for OECD

APPENDIX C NEUROTOXICITY TESTING METHODS

C.1. BEHAVIOURAL TESTS

C.1.1. Functional observational battery

Functional Observational Battery (FOB) is a standardised screening battery for the assessment of many aspects of behaviour and neurological functions, usually in rodents. Many forms of FOB have been described (Irwin, 1968; Gad, 1982; Alder and Zbinden, 1983; Moser *et al*, 1988; Schulze and Boysen, 1991). Common to all is the intention to detect gross functional deficits and to evaluate qualitatively general appearance, behaviour, general toxicity, muscle weakness, sensory function and reflexes (examples of behavioural procedures included in a FOB are given in Appendix Table C-1).

The FOB evaluates respondent behaviour which is the simplest form of behaviour, elicited by known, observable stimuli. Although respondent behaviour is very reproducible and relatively easily quantified, the robustness of the behaviour means that many of the tests may not be sensitive to subtle changes. Furthermore, the assessment is very subjective, often descriptive and depends on the observer. A further shortcoming of the FOB is that the battery does not adequately assess functional reserve capacity and could result in false negative findings. No single set of standardised tests will detect all possible health hazards. Several test batteries have been validated by comparing the effects of known neurotoxic chemicals such as acrylamide and 3,3'-iminodiproprionitrile (Schulze and Boysen, 1991), methylmercury, chlordecone and organotin (Pryor *et al*, 1983), organoleads (Walsh and Tilson, 1984), chlordimeform and carbaryl (Moser *et al*, 1988), 3-acetyl pyridine, pentobarbital, nicotine and triadimefon (Moser and MacPhail, 1990).

The use of a FOB as a standardised screening battery may help to identify neurotoxic chemicals, to produce a profile of effects for comparison with other chemicals, and to aid in making decisions regarding further testing. Many observations in a FOB such as convulsions, tremor, ataxia, irritability and others correspond to symptoms or signs most commonly reported in human beings who have been exposed to neurotoxic substances (Moser, 1990).

C.1.2. Motor function and motor activity

Deficits in motor function can be specifically measured by several test systems including rotating rods, inclined planes, and assessment of swimming ability (Spyker *et al*, 1972; Robbins, 1977; Bogo *et al*, 1981; the most common techniques for measuring motor function are listed in Appendix Table C-2). Normally, these are very simple techniques

and animal training is frequently not required. A disadvantage of these methods is the variance in the data, since the ability or willingness of individual animals is likely to range considerably, and this makes most of the measurements insensitive. Validation has been made with substances such as methylmercury (Spyker *et al*, 1972), lead (Bornschein *et al*, 1980), acrylamide and ethanol (Bogo *et al*, 1981).

Motor activity is an operant or emitted response which is not elicited by a single identifiable stimulus, but occurs within the context of many environmental stimuli. Furthermore, it is not a single activity but consists of many acts, such as walking, rearing, sniffing, grooming, and tremor (Reiter and MacPhail, 1979; Rice, 1990). For this reason, a huge variety of different methods of measuring motor activity have been described and used (Appendix Table C-2). There are several advantages of motor activity testing since its recording is non-invasive and most methods provide a quantitative assessment of locomotor activity which may be analysed statistically. Furthermore, computer technology has allowed the evaluations to be predominantly automated; it has greatly increased the amount and quality of data that can be obtained from locomotor activity measures of laboratory animals (Reiter, 1978; Evans *et al*, 1986) and eliminated the potential influence and bias of the observer. The measurement of motor activity as a behavioural end-point has certain limitations. Altered motor activity may not directly reflect the neurotoxic action of a compound since several external and internal factors including apparatus, relative novelty and complexity of the environment, nutritional and hormonal state, biological rhythms, age and social setting can markedly influence activity levels (Strong, 1957; Robbins, 1977; Reiter, 1978). Moreover, specific measurements of motor activity such as recording by photocells without further differentiation have been shown to be relatively insensitive to neurological damage (Pryor *et al*, 1983; Gerber and O'Shaughnessy, 1986). In addition while a lesion can affect motor activity in a reasonably predictable manner, the reverse is not true. A general decrease or increase of motor activity does not yield any information about lesion site or brain region involved. A change in motor activity always requires further testing to evaluate the significance but does not provide the information about which test ought to be conducted (Maurissen and Mattsson, 1989).

The assessment of motor activity on its own is inadequate as an indicator for neurotoxicity. Only in the absence of systemic toxicity or other confounding factors can a dose-related change in motor activity reflect an effect on the nervous system.

C.1.3. Sensory function

A variety of techniques, from very simple to extremely sophisticated, has been utilised to assess sensory functions in animals exposed to neurotoxicants (Appendix Table C-2.).

Specific testing of sensory functions after exposure to neurotoxicants is required, first to exclude impaired sensory function as a confounding factor for other neurobehavioural tests, second because there are several chemicals such as acrylamide (Merigan *et al*, 1982), 3,3'-iminodipropionitrile (Peele *et al*, 1990) or aminoglycosidic antibiotics (Stebbins and Rudy, 1978) which affect sensory function before producing other signs of toxicity and third, there are sensory deficits which occur without external physical evidence (Evans *et al*, 1975). In addition, sensory deficits such as impairment of scotopic vision may be one of the earliest indicators of central nervous system intoxication (Evans, 1982). The evaluation of toxicity in sensory systems presents particular problems because of the need to distinguish specific sensory loss from other toxic effects.

A technique for the discrimination of sensory from non-sensory toxic effects is the modulation of reflex startle by presentation of a low intensity stimulus immediately prior to a high intensity stimulus that elicits the startle response (Hoffman and Ison, 1980; Ison and Hoffman, 1983). The assessment of modulation of reflex behaviour by low-intensity test stimuli is more sensitive than the assessment of the ability of high-intensity stimuli to elicit a reflex (Fechter and Young, 1983).

In addition, animals can be trained e.g., by means of an intermittent schedule of reinforcement (cf Appendix C.1.4.) to report reliably about their sensory perception. Thus, auditory (Stebbins and Rudy, 1978) and somatosensory thresholds (Maurissen *et al*, 1983) as well as visual impairment (Merigan *et al*, 1982) can be detected. Very often, such testing is carried out with monkeys as they have sensory function similar to man. Other species, in particular, rodents and birds, may be appropriate depending on the experimental conditions (Evans, 1982). Rats and pigeons can be trained to discriminate between two stimuli e.g., different luminances, to give information on visual impairment (Blough, 1957; Friedman and Carey, 1978). Thus, very subtle sensory deficits can be demonstrated.

Assessment of sensory functions is an indispensable aspect of neurotoxicity testing in cases of structure-activity relationship to other sensory neurotoxicants or if sensory impairment is indicated by previous tests. Furthermore, it is necessary to exclude selective impairment of sensory functions to identify possible confounding factors for other neurobehavioural tests.

C.1.4. Learning and Memory

Learning can be defined as an adaptation to changes in the environment and memory as the retention of such an adaptation over some time span. These two processes can never

be considered in isolation (Cabe and Eckerman, 1982). An agent may affect learning competence, performance capability for particular tasks, or both. Therefore, before concluding that learning competence has been compromised, the ability to perform the criterion tasks must be determined. Measures of acquisition are influenced by non-associative factors, including sensory, motor and motivational variables (Tilson *et al*, 1980; Pryor *et al*, 1983), thus, these factors must be excluded by independent assessments or by including specific indicators of their function (Eckerman *et al*, 1980). Some common techniques to measure learning and memory ability are listed in Appendix Table C-2.

Flavour-aversion conditioning is based on the animal's ability to avoid a substance ingested shortly before the onset of an adverse effect. The preferences of rats for distinctly flavoured solutions e.g., saccharin, can be markedly reduced by pairing intake with administration of neurotoxic substances such as trialkyltin compounds and 3,3'-iminodipropionitrile (MacPhail, 1982; Peele *et al*, 1990). This conditioned taste aversion can be produced at doses below those reported to affect other behaviour patterns. Furthermore, in this test motor function and activity level are not major confounding factors. It is difficult to determine whether the results of the test are a consequence of changes in cognitive function or due to altered gustatory sensitivity. In addition flavour aversion is not specific for neurotoxicants but can also be induced by chemicals affecting systemic organs and even at non-toxic doses by psychopharmacologically active substances.

A commonly used procedure for the assessment of cognitive function is active or passive avoidance. Active avoidance requires the animal to leave a specific area at the onset of a cue to avoid an aversive stimulus such as electic shock (Sobotka *et al*, 1975). In a passive avoidance situation the animal must remain in the specified area to avoid shock. Passive and active avoidance procedures are considered rather nonspecific because they are affected by arousal and sensomotor function of the animal and thus, conflicting results are often obtained (Costa and Murphy, 1982; Rice, 1990).

Discrimination tasks are often used to study the effects of neurotoxicants on learning and memory. The animal has to choose between two or more stimuli which are simultaneously presented. In a spatial discrimination procedure the animal must decide a direction or spatial position in order to receive a reward. Since rodents have a natural predilection for running through confined areas it is common to evaluate their performance in mazes such as T-maze, radial arm maze, or water maze (Cabe and Eckerman, 1982).

In the T-maze, one of the simplest examples of spatial tasks, the tendency of rats to alternate spontaneously the goal arms can be used. Rats usually enter the arm which

was not visited on the previous trial (Olton, 1979). The radial arm maze consists of a platform from which 4 to 24 arms radiate. Rats are allowed to choose freely among the arms but only one piece of food is available at the end of each. The optimal strategy is to choose each arm once without repeating any arm (Olton, 1979). In the water maze, the rat must swim to a submerged platform in a large tank filled with opaque water (Rogers and Tilson, 1990). All these test systems assess working and reference memory. The hippocampus and its cholinergic input are an important neurobiological substrate for cognitive processes such as spatial memory. Thus, damage to this brain region by neurotoxicants, including heavy metals and their alkyl derivatives (Walsh and Chrobak, 1987; Walsh and Emerich, 1988), organophosphates (McDonald *et al*, 1988), the cholinergic neurotoxin AF64A (Chrobak *et al*, 1987), and excitatory amino acids (Milgram *et al*, 1988; Rogers and Tilson, 1990), leads to a transient or persistent impairment in cognitive function which can be measured using mazes. Nevertheless results can be confounded by disturbed motor or sensory function.

Common non-spatial discrimination task use the operant conditioning chamber where the animal responds by poking its nose into a hole or by pressing a lever or key (Levine *et al*, 1977; Winneke *et al*, 1977; Cabe and Eckerman, 1982). These tasks have proved to be sensitive to impairment resulting from exposure to heavy metals (Winneke *et al*, 1986). Once the task is learned, a discrimination reversal paradigm provides additional information on the animal's learning ability by requiring the animal to learn a new task. Chronic exposure to lead has been found to produce deficits in discrimination reversal in monkeys (Rice, 1985).

A test of attention and short-term memory is "matching to sample". Various species, including pigeons and monkeys can be used (McMillan, 1981; Rice, 1984). The animal is presented with a stimulus such as a colour, pattern, or an object that is then withdrawn. Afterwards, a set of stimuli are presented, and the animal has to indicate which of these is identical to the sample stimulus. Some delay may be inserted between the presentation of the sample and test stimuli to test short-term memory. Several substances, including pentobarbital, phencyclidine, morphine, amphetamine, tetrahydrocannabinol (McMillan, 1981), and lead (Rice, 1984) have effects in such a delayed matching to sample task. However, intact functioning of sensory and motor behaviour is required.

In an operant task with an intermittent schedule of reinforcement, the animal is not reinforced for every response but for a number of responses according to certain rules (Rice, 1990). There are a number of procedures of reinforcement schedules, such as the fixed ratio (FR) schedule which requires the animal to emit a fixed number of responses in

order to be reinforced, or the fixed interval (FI) schedule which requires that a fixed length of time to elapse before a response is reinforced. Intermittent schedules may also be maintained by negative reinforcement, usually a short electric shock. Simple intermittent schedules have been widely used and have proved to be sensitive to the effects of a number of industrial and environmental toxicants, including methyl n-amyl ketone (Anger *et al*, 1979), lead (Dietz *et al*, 1978; Cory-Slechta *et al*, 1981), and chlordimeform (MacPhail and Leander, 1981). However, responding to schedules of reinforcement may be affected by impaired motor function or a decrease in motivation, and thus, effects other than direct effects on the nervous system may be observed (Tilson *et al*, 1980; Weiss *et al*, 1981). Additionally operant performance usually requires food or water deprivation, and time for training the animals to establish a stable level of performance. Furthermore there is no consistent relationship between schedule-controlled operant performance and neurotoxicity since not all neurotoxic compounds cause changes in operant behaviour (Levine *et al*, 1980) whereas many therapeutically useful drugs have clear effects on this kind of behaviour without being neurotoxic (Rastogni and McMillan, 1984). Thus, operant performance may be more valuable in characterising the actions of identified neurotoxicants as well as evaluating possible mechanisms of action (Moser and MacPhail, 1990).

In conclusion, comparable to the assessment of sensory functions, evaluation of cognitive functions is a valuable tool to further characterise a neurotoxic effect involving the central nervous system. Because performace in tests of learning and memory can be affected by many confounding factors, data should be interpreted very carefully.

C.2. ELECTROPHYSIOLOGICAL TESTS

In this section a variety of electrophysiological methods are briefly described and examples of their use given. It is not the intention to describe all electrophysiological techniques but rather to include those which have been used with varying degrees of success in detecting neurological damage. Reviews of the methods described are referenced and described below.

C.2.1. Electroencephalography

The electroencephalogram (EEG) is a measure of the dynamic process of the instantaneous integrated activity of the brain, which represents ongoing processes under higher nervous control. Changes in frequency, amplitude, variability and pattern of the EEG are thought to be directly related to underlying biochemical and physiological

changes (Fox *et al*, 1982). Descriptions and discussions of EEG techniques have been reviewed by Benignus (1969, 1982, 1984) and others (Johnson, 1980; Fox *et al*, 1982).

The EEG is easily recorded from a variety of species, including man, using non-invasive scalp macroelectrodes, although in rodents the electrodes are usually implanted into the skull. The electric potential differences between the electrodes are measured and the change in the potential difference recorded. The EEG is readily quantified, samples several regions of the brain simultaneously (it has been reported by Nunez (1981) that such macroelectrodes measure the space-averaged activity of 10^7 or more neurons), requires a minimum of cooperation from the subject and is generally unaffected by previous testing. As the EEG is dynamic it reflects instantaneous changes in the state of the CNS and thus to a great extent the state of arousal or anaesthesia (Jouvet, 1967; Fox *et al*, 1977).

As the EEG recorded using scalp electrodes is a summation of the cortical activity beneath the electrode it may not accurately reflect non-cortical lesions, although this may be partially overcome by utilising techniques which change the pattern of the EEG, such as hyperventilation (which can activate slow wave abnormalities), stimulation producing arousal (light, sound, electrical stimulation), or sleep (Desi and Sos, 1962, 1963; Cooper *et al*, 1974; Seppalainen, 1975). Alternatively, the EEG may be recorded using electrodes implanted directly in specific brain structures. These have particular patterns of activity which can be quantitatively examined. For example, hippocampal discharge has been examined in depth by Dyer *et al* (1979a, b) and hippocampal EEG by Naasland (1986).

A number of disadvantages limit the use of the EEG. It cannot provide information on the integrity of specific motor or sensory pathways. The data produced can be difficult to interpret and, as it does not provide information at the cellular level, it cannot be used to provide details on mechanism of action (Johnson, 1980; Arezzo *et al*, 1985; Seppalainen, 1988).

In clinical neurology the EEG has been used for the diagnosis and description of epilepsy and many other neurological disorders (Niedermeyer and Lopes da Silva, 1982). It has also been used in neurotoxicological assessments (Eccles, 1988) and changes in the EEG have been reported in animals and man following exposure to toluene (Takeuchi and Hisanaga, 1977; Hisanaga and Takeuchi, 1983), organophoshates (Duffy *et al*, 1979; Nagymajtenyi *et al*, 1988) and pesticides (Desi, 1983). The full potential of the technique in neurotoxicology has not yet been fully exploited (WHO, 1986). The EEG is, nevertheless, a useful and recommended clinical test in the evaluation of neurological diseases and in occupational medicine, particularly in cases in which accidental exposure

produces symptoms of CNS involvement (Seppalainen, 1975, 1988) and is an integrated part of Soviet and Eastern European neurotoxicological studies (WHO, 1986).

C.2.2. Evoked Potentials

Electrical potentials recorded from the brain in response to external sensory stimuli are called sensory evoked potentials (EPs). Their use in neurotoxicology has been reviewed by Johnson (1980), Fox *et al* (1982), Rebert (1983), Dyer (1985, 1987) and Mattsson and Albee (1988). Sensory EPs are especially useful clinical and research tools for evaluating integrated sensory responses in the CNS. They have been particularly valuable in detection of clinically silent lesions such as multiple sclerosis (Halliday *et al*, 1973; Asselman *et al*, 1975; Mastaglia *et al*, 1976, 1977) and to monitor difficult patient populations such as comatose patients and children (Bodis-Wollner, 1982).

The types of EP most commonly recorded in laboratory animals are visual (VEP or VER), auditory (AEP) and somatosensory (SSEP). VEPs, which include flash evoked potentials (FEPs) and pattern reversal evoked potentials (PREPs), are used to evaluate the effects of substances on the components of the nervous system responsible for vision. Potentials can be generated using stimuli ranging from diffuse light flashes to complex patterns of shape and colour (Dyer, 1985; Mattsson and Albee, 1988; Otto *et al*, 1988). If abnormalities in FEP are observed electroretinograms, recorded using corneal electrodes, may be used to aid in the interpretation (Rebert, 1983). Auditory EPs may be recorded from the cortex or the brainstem (BAER) in response to clicks and can be used to detect specific losses in the auditory system. To assess peripheral auditory dysfunction more adequately the BAER may be generated using tone pips of varying frequencies and intensities (Rebert, 1983). SSEPs are elicited by low amplitude electrical stimulation of peripheral nerve. The ventral caudal nerve of the tail in rats usually is stimulated while EPs are recorded from the somatosensory cortex. Recording from the cerebellum can help to differentiate effects and/or more precisely localise lesions. The SSEP records activity from the entire sensory pathway from the limbs to the brain (Mattsson and Albee, 1988; OTA, 1990). Early components of the SSEP are thought to represent far-field dorsal column and thalamic activity, and later components are of near-field cortical origin (Wiederholt and Iragui-Madoz, 1977; Arezzo *et al*, 1979)

EPs have a number of advantages for use in laboratory animals which include a fixed temporal relationship to the evoking sensory stimulus. In this respect they are extremely reliable both within and between individuals allowing quite minor changes to be detected; and, in some cases, they are similar even among several species enabling more accurate extrapolation (Hudnell *et al*, 1990). They also reflect rather directly the integrity of the

sensory CNS pathways they can yield specific information about particular neuroanatomical pathways and allow correlations of structure and function (Dyer and Annau, 1977; Dyer *et al*, 1978; Fox, 1979; Mattsson *et al*, 1988). They can be recorded simultaneously with behavioural measures and, hence, the neurological significance of some neurobehavioural changes can be assessed (Rebert, 1980). EPs can be recorded non-invasively, and can, therefore, be used to monitor the progress of neurotoxic damage and/or recovery in the same individual (Rebert *et al*, 1982). EPs are also well suited to the study of very young animals because of the difficulty of assessing sensory capacity in other ways. Albee and Mattsson (1983) have described a reliable method of VEP recording in rats as young as 10 days of age.

EPs are not without interpretational difficulties as they may be altered by a variety of factors such as toxic reactions, temperature, hypoxia, sensory deficits, central dysfunction and vitamin deficiency (Rebert, 1983; Albee *et al*, 1987). It is sometimes difficult, therefore, to discriminate a direct neurotoxic effect from other consequences of treatment. In addition, sensory evoked potentials evaluate only selected pathways in the CNS and it is known that the shape of later components of the potentials reflect "cognitive" function, and as such are affected by a number of psychological variables (Begleiter, 1977; Standage and Fleming, 1979; Ollo and Squires, 1986). Nevertheless, if one accepts that normal physiology and brain function are inseparable (Black *et al*, 1987), then higher brain functions are unlikely to be abnormal and yet have normal electrophysiological responses from the visual, auditory and somatosensory systems (Mattsson and Albee, 1988).

EPs have been included in a large number of neurotoxicology studies (Seppalainen *et al*, 1981; Rebert, 1983; Dyer, 1985; Mattsson and Albee, 1988) although many studies used only the FEP. It is clear from the evoked potential literature as a whole that different chemicals can produce different profiles of sensory effect in the nervous system. For example, mercury and carbon disulphide appear to influence FEPs more than SSEPs (Lehotzky and Meszaros, 1974; Dyer *et al*, 1978; Rebert and Becker, 1986; Rebert *et al*, 1986). The SSEP, however, appears to be particularly sensitive to the effects of lead poisoning (Seppalainen, 1978). Thus, in the attempt to use EPs to determine whether or not a chemical is neurotoxic, it may be necessary to perform a fairly comprehensive multisensory examination (Rebert, 1983).

Studies with EPs confirming functional deficits in pathways known to be histopathologically damaged have proved the validity of the methods. For example, in hexachlorophene treated animals SSEPs confirmed the area of damage to be peripheral nerve and spinal cord (Mattsson *et al*, 1989), and BAERs confirmed toluene induced deafness (Pryor and

Rebert, 1983). In addition, at least one form of neurotoxicity was first described as a result of an electrophysiology study. Dyer and Howell (1982) revealed effects in FEPs after exposure to triethyltin although previous studies specifically excluded the retina as being damaged. Following additional recording from the optic tract, indicating changes at the retinal level, a closer histopathological examination of the retina revealed a loss of retinal ganglion cells.

EPs have been shown to be useful indicators of neurotoxicity in animals (Rebert, 1983) although the applicability to neurotoxicology has only recently been accepted. Nevertheless, the rapid improvement in techniques and increasing demonstrations of validity suggest that EPs will become more important to neurotoxicology in years to come.

C.2.3. Peripheral Nerve Conduction Velocity Measurements

The most commonly used test of electrophysiological function in neurotoxicology is the determination of the conduction velocities in peripheral nerves (Johnson, 1980). These are relatively rapid and simple to perform and can be performed non-invasively. Similar to the EEG and EPs the data can be directly extrapolated to corresponding studies in man (Bergmans, 1983). Clinical signs and symptoms of peripheral nerve dysfunction can frequently be correlated with abnormal conduction in groups of motor and sensory nerve fibres (Gilliatt and Willison, 1962; Gilliatt, 1966).

Conduction velocity is measured by eliciting a nerve impulse, through electrical stimulation at two different sites of a particular nerve, and recording the nerve action potential at some distance from the stimulation sites. Nerve conduction velocity is calculated by measuring the distance between the two sites of stimulation and dividing by the difference in the two latency times (Misumi, 1979). For motor nerves, recording the action potential of the muscle innervated by the nerve under investigation measures the motor nerve conduction velocity (MNCV) independently of the delay at the myoneural junction, and thus more accurately measures effects on the nerves. Nerve conduction velocity measurements are usually made from mixed (motor and sensory) nerves. Both sensory (SNCV) and motor nerve conduction velocities can be recorded from the same nerve by stimulating the nerve in two directions (Misumi, 1979) as nerves conduct impulses slightly faster in their natural direction.

Electrophysiological tests may be performed *in vitro* on excised nerves (Birren and Wall, 1956) or *in vivo* using surgically exposed nerves (McDonald, 1963; DeJesus *et al*, 1978) or in the intact animal (Miyoshi and Goto, 1973; Glatt *et al*, 1979; Misumi, 1979; Schaeppi *et al*, 1984). Non-invasive techniques have been described for a number of species

including the rat, in which the tail nerve is used (Miyoshi and Goto, 1973; Glatt *et al*, 1979; Misumi, 1979;) and the dog using chronically implanted cortical electrodes (Schaeppi and Krinke, 1982, 1985; Schaeppi *et al*, 1984).

The procedures for measuring conduction velocities in the intact animal are of considerable advantage as tests can be performed on the same animal throughout the course of the study (Goto and Peters, 1974; Rebert *et al*, 1982). This allows the technique to be used in toxicity studies without the need for additional animals. A difficulty in determining conduction velocities in intact animals is the accurate measurement of the conducting distance. In the rat, tail distances can be measured with sufficient accuracy. For other nerves, such as the sciatic, the uncertainty of electrode location and of the anatomical path introduces an error into the measurements and, accordingly, the calculated conduction velocity. The variability can be reduced by using preparations in which the nerve is surgically exposed, but in this case different animals need to be used to follow the progress of defects following intoxication (McDonald, 1963; DeJesus *et al*, 1978).

A further difficulty with nerve conduction velocity measurements is that conduction velocity is usually calculated as maximum velocity, and hence represents the conduction velocity of the fastest conducting axons. Effects on slower conducting axons can be difficult to detect. Damage to slow conducting fibres may not be reflected as a change in conduction velocity but rather as a decrease in action potential amplitude (DeJesus *et al*, 1978). Another important consideration when conducting nerve conduction velocity measurements is that nerve conduction velocity varies with temperature (Birren and Wall, 1956; DeJong *et al*, 1966) and it is of importance to ensure that the temperature of the nerve is monitored and/or remains constant.

A major difficulty in recording sensory nerve conduction velocities (SNCVs) is that many of the sensory functions affected in neurotoxicity such as pain, perception of pain and temperature, are mediated by very small diameter fibres from which signals are more difficult to monitor (Fox *et al*, 1982).

Conduction velocity measurements, recorded either *in vivo* or *in vitro*, have been used in a large number of neurotoxicology studies involving a wide variety of species and confirmation of electrophysiologically detected peripheral nerve damage by histology have proved the value of such measurements. For example, Fullerton (1966), Fullerton and Barnes (1966) and Leswing and Ribelin (1969) demonstrated reduced MNCV in rats, guinea-pigs, cats and monkeys following acrylamide intoxication was associated with segmental demyelination and axonal degeneration; experimental diabetic neuropathy in

the rat was monitored using MNCV and SNCV measurements (Miyoshi and Goto, 1973. Goto and Peters, 1974); changes in peripheral nerve conduction velocity were correlated with histological changes in rats with hexachlorophene neuropathy (Maxwell and Le Quesne, 1979), cats with diphtheritic polyneuritis (McDonald, 1963) and in dogs with pyridoxine or organophosphate-induced neuropathy (Schaeppi and Krinke, 1982; Schaeppi *et al*, 1984). The absence of neurotoxicity has also been demonstrated in studies involving conduction velocity measurements. For example, two chemicals previously suggested to be neurotoxic, methylene chloride and 2,4-dichloroohenoxyacetic acid, were shown to be without effect on a variety of neurotoxicological measures including peripheral nerve conduction velocity (Mattsson *et al*, 1986, 1990a).

Peripheral nerve conduction velocity measurements are relatively simple and quick to implement and can be easily incorporated into routine toxicity studies. They are useful measurements for neurotoxicological investigations, particularly where peripheral neurotoxicity is suspected. The methods are also routinely included in neurotoxicological investigations in man allowing relevant direct extrapolation from animal studies.

C.2.4. Electromyography

The electromyogram (EMG) is an extracellular recording of the electrical potential of muscle and is particularly useful for examining neuromuscular deficits (WHO, 1986). Electromyograms are recorded with electrodes which are either placed on the skin above the muscle or, more usually, by needle electrodes inserted into the muscle (extracellularly). The electrical activity evoked by insertion and movement or the needles, the electrical activity of the resting muscle, and that of motor units during voluntary muscle contraction are measured (Johnson, 1980).

Electromyography is often used to investigate direct toxic effects on muscles and evoked muscle responses to nerve stimulation are invaluable in examining the neuromuscular junction (WHO, 1986). Neuromuscular transmission can be affected by various neurotoxic agents, such as botulinum toxin and organophosphate insecticides. Altered neuromuscular function in organophosphate insecticide workers who did not exhibit any other signs of poisoning were detected by EMG (Drenth *et al*, 1972; Roberts, 1977).

EMG has been used extensively in human clinical studies in the diagnosis of certain myopathies and neuropathies (Licht, 1961) but only occasionally in neurotoxicity studies involving experimental animals. The main reasons for the limited use of the EMG are: one component of the EMG involves the voluntary graded contraction of the muscle being evaluated which can be difficult in laboratory animals; and secondly the technique requires

significant cooperation from the animal to record from a muscle at rest unless anaesthesia is used. Nevertheless, the EMG has been used successfully in neurotoxicological studies. Mendell and co-workers (1974) demonstrated EMG abnormalities in all muscles of cats exposed to methyl n-butyl ketone, with more marked changes occurring distally. These findings are consistent with muscle denervation due to distal axonopathy. Ulrich *et al* (1979) investigated the effects of manganese dust exposure in monkeys and were able to define an exposure level for manganese dust which does not cause neurotoxicity.

EMG methods are available for use in experimental animal neurotoxicology investigations although limited cooperation of animals may limit its application. When indicated by other data, the use of this technique can provide a functional picture of an animals neuromuscular status that is as complete as possible (Johnson, 1980).

C.2.5. Intracellular Recording and Other Applied Electrophysiology Techniques

Electrophysiological techniques such as intracellular microelectrode recording, iontophoresis and voltage clamp enable cellular mechanisms of action of neurotoxicants to be determined. These techniques, including recent developments such as patch clamping for the investigation of individual ion channels, have been reviewed extensively by Kerkut and Heal (1981) and Atchison (1988).

The techniques enable detailed analysis of synaptic processes including neurotransmitter storage and release, receptor activation and ionic fluxes (Atchison, 1988). Electrical potential parameters such as resting membrane potential, spike amplitude and end-plate potential frequency can be measured and changes in nerve function such as altered excitability and ability to fire repetitively can be analysed. From this deductions can be made about the mechanism of action of a neurotoxicant.

Intracellular recording involves the insertion of small diameter (<2 μm), usually glass, microelectrodes into individual nerve or muscle cells and is, therefore, particularly suited to *in vitro* preparations. Isolated central nervous system preparations from cold-blooded vertebrates have been extensively investigated using electrophysiological techniques (Rowan, 1985). Although relatively few neurotoxicological studies have used *in vitro* methods these are now receiving more attention (Kerkut and Heal, 1981). The types of *in vitro* preparation suited to these techniques are described below (cf Appendix C.7.)

The main advantage of these types of methods is the precision of the information they provide. This is achieved by allowing the study of information transfer between and within excitable cells in real time; the dissection of synaptic transmission into its components; and the ability to study subtle, early changes (Atchison, 1988). Disadvantages of the

techniques are in determining the relevance of the observed cellular effects to neurotoxic signs manifest in the whole animal, and the level of expertise needed to perform, analyse and interpret the results (Atchison, 1988).

in vivo intracellular recording from dorsal root ganglion or anterior horn cells of terminally anaesthetised animals have been utilised, although infrequently, in neurotoxicology studies. Somjen *et al* (1973) recorded from dorsal root ganglion cells of rats intoxicated by methylmercury and showed abnormal sensory function. This work also showed the dorsal root ganglion to be an initial focus for methylmercury neurotoxicity.

The methods are not applicable for the screening of neurotoxicity as only specific areas of the nervous system can be examined in any experiment. They have greatest utility in mechanistic studies in the final resolution of the actions of selected neurotoxicants (WHO, 1986; Atchison, 1988; OTA, 1990).

C.3. NEUROCHEMICAL TESTS

The main problem inherent to a neurochemical approach of neurotoxicity screening is the wide variety of direct and indirect mechanisms underlying neurotoxicity. General aspects thereof have been discussed in Section 4.2.3. Some of the mechanisms of neurotoxicity for specific chemicals are listed in Appendix Table C-3.

The aim of this section is to review some of the neurochemical approaches that may be useful for identifying neurotoxic effects and to shed some light on the inherent difficulties in interpreting the data.

C.3.1. Neurotoxic Esterase (or Neuropathy Target Esterase; NTE)

Some organophosphates are known to produce a delayed neuropathy (organo-phosphate-induced delayed neuropathy, OPIDN) after animals have overcome the acute cholinergic effects induced by the compound. OPIDN is precipitated by prolonged inhibition of neurotoxic esterase. Transient inhibition of NTE, even if total, is not sufficient to induce delayed neuropathy (Johnson, 1982). Only prolonged inhibition over a threshold level of 70 - 80%, as a result of covalent binding of the organophosphate to NTE (aging), results in OPIDN (Johnson, 1982).

Neurotoxic esterase is a membrane bound protein whose biological function is not known. Also the sequence of molecular and cellular events triggered by the inhibition of NTE and ends with the degeneration of certain long axons about 10 days later has still to be elucidated (Abou-Donia and Lapadula, 1990; Johnson, 1990). To predict the occurrence

of OPIDN the activity of NTE is usually determined in tissue homogenates 48 hours after the last dose of an organophosphate (Johnson and Richardson, 1983).

The concept that inhibition of NTE and subsequent aging leads to delayed neuropathy is now widely accepted and has been confirmed for all but one neurotoxic organophosphate (Capodicasa *et al*, 1991). When testing for delayed neurotoxicity following acute administration measurement of NTE can contribute to the identification of a possible hazard in cases where clinical signs are negative or equivocal. A second administration in the same animals and/or a delayed neurotoxicity testing following repeated administration can thereby be eliminated.

C.3.2. Biochemical Markers for Neurons or Glial Cells

Some molecules are present in only one or few cell types of an organ or tissue as e.g., structural proteins, glycoproteins, enzymes, lipo-proteins etc. In general the concentration of such cell type specific molecules is regulated in a rather narrow band. Cell type specific molecules can thus be used as biochemical markers for the presence or absence of these cells. By using quantitative or semi-quantitative methods changes in the number of cells from which they originate can be estimated. In general, biochemical, electrophoretic or immunochemical methods are used to detect and quantify marker molecules. Such investigations may be helpful in assessing cell degeneration or reactive gliosis, but in general are poor predictors of functional integrity of cells.

Peripheral myelinopathy is characterised by selective degeneration of Schwann cells. In contrast, Wallerian degeneration of peripheral axons (Waller, 1852) is accompanied by concomitant loss of Schwann cells that form the myelin sheaths. Both neuropathies usually are accompanied by a transient invasion of macrophages. Loss of one or both of the major cell types of peripheral nerves as result of a toxic insult is reflected in decreases in their respective marker molecules (axon-specific S-100 protein and/or myelin X glycoprotein) and a transient increase in the macrophage marker enzymes β-galactosidase and β-glucuronidase (Dewar and Moffett, 1979; Miyake *et al*, 1989).

In the central and peripheral nervous system methylmercury, at repeated doses that induced partial hind-limb paralysis, induced changes in thyrosin hydroxylase activity in the striatum and in glial markers glutamine synthetase and 2',3'-cyclic nucleotide-3-phosphohydrolase in optic and sciatic nerves (Kung *et al*, 1989). Repeated exposure to toluene induced dose-dependent changes in γ-enolase (neuron-specific marker) and α-enolase, β-S100 protein and creatine kinase-B (glial-specific markers) in various brain regions investigated (Huang *et al*, 1990).

In the central nervous system neuronal degeneration is followed by a reactive gliosis that is correlated with a decrease in synapsin, a neuron-specific protein. After dosing rats with trimethyltin a reactive gliosis measured as an increase in glial fibrillary acidic protein (GFAP) was observed in hippocampus and frontal cortex (O'Callaghan, 1988). Since reactive gliosis is a process common to all central neural lesions the GFAP assay may detect lesions induced by a neurotoxicant. The specificity and reliability of this assay has not yet been adequately evaluated to allow this test to be used as a standard assay.

Depending on the cell type, marker molecule, and type of toxic insult, changes in marker molecules can be permanent or transient (Dewar and Moffett, 1979; O'Callaghan, 1988). In order not to miss an effect, measurements have to be performed at several time points during or after exposure in order to detect changes. This need for multiple measurements limits the usefulness of some marker molecules as a tool for a basic neurotoxicity screen. In addition, biochemical determination of such marker molecules is usually performed in fresh tissue and increases the number of animals needed for safety evaluation of chemicals. When used as histochemical or immunohistochemical staining techniques marker molecules can be very helpful in localising or further characterising a lesion.

A list of some of the most common cell-type specific marker molecules, their corresponding cell type and their subcellular localisation is given in Appendix Table C-4.

C.3.3. Biochemical Parameters Related to Signal Transmission

Neurotoxic agents such as lead (Shih and Hanin, 1979; Silbergeld, 1985), manganese (DiStefano and McKenna, 1977), organochlorinated or organophosphorous pesticides (Hrdina *et al*, 1973; Karczmar, 1984) have been reported to interfere with different aspects of neurochemical transmission. Due to the ease of measuring tissue concentrations of neurotransmitters, most of the studies focused on steady state levels of neurotransmitters. Such steady state levels, however, are difficult to interpret particularly when they are measured in homogenates of whole brains or of large brain areas (Damstra and Bondy, 1982).

Neurotransmitters can be stored in different pools that have different availability (Taylor and Brown, 1989) and steady state levels can be relatively unaffected despite marked fluctuations in activity of the neurons (Cooper *et al*, 1986; Weiner and Molinoff, 1989). Increased activity in a neurotransmitter system is correlated with increased synthesis and turnover rates of its transmitter. Furthermore, to maintain functional homeostasis during prolonged stimulation, neurotransmitter systems react with downregulation of their postsynaptic receptors (Schlichter, 1979; Damstra and Bondy, 1982; Cooper *et al*, 1986).

Thus, turnover rates of neurotransmitters, activity of the rate-limiting enzymes or receptor binding studies are more relevant measures for the activity of specific neurotransmitter systems (Damstra and Bondy, 1982).

The interpretation of neurotransmitter- or receptor-related data is further complicated when testing neuropharmacologically active compounds. Due to their direct action on neurotransmitter systems, changes in neurochemical parameters are not necessarily predictive of a neurotoxic effect, but may be related to their neuropharmacologic action (Eibergen and Carlson, 1976; Ben-Barak *et al*, 1981; Nimgaonkar *et al*, 1985).

These problems limit the usefulness of biochemical markers of neurotransmittor functions in a basic neurotoxicity screen. As stated for cell specific marker molecules such measurements may be helpful in further characterising a neurotoxic effect or in determining a NOEL.

C.3.4. Biochemical Parameters related to Cell Functions or Cell Integrity

Basic functions of whole cells or cell organelles such as glucose phosphorylation, synthesis of ATP, NADH, RNA or proteins or maintenance of ionic gradient across membranes may be measured to assess cytotoxic effects in *in vitro* studies. Since these parameters characterise basic cellular functions, they can also be used to determine the viability of nerve or glial cells. Normal metabolism of neurons or glial cells is a prerequisite of their normal function and thus of the intact nervous system.

Determination of such parameters has successfully been used to further characterise neurotoxic effects or to determine a NOEL. Neurotoxic effects can be reflected in altered local glucose utilisations (Orzi *et al*, 1988), decreased RNA or protein synthesis (Schotman *et al*, 1978; Albrecht, 1984), changes in energy-linked functions (Lai *et al*, 1980; Husain *et al*, 1986; Sickles and Goldstein, 1986) or increases in myo-inositol-1-phosphate (Honchar *et al*, 1983).

Changes in these parameters may also be observed as a result of normal neural activity during sensory stimulation (Sharp *et al*, 1981), states of altered activity or emotion (Roland *et al*, 1982; LeDoux *et al*, 1983), pharmacological action of a chemical (Sokoloff, 1981; Muller and Martin, 1984) or may be absent in the presence of neurotoxic effects (Maier and Costa, 1990). In addition, nervous system function may be disrupted in the absence of disturbed basic cell functions by interference with mechanisms related to signal transmission such as receptor functions, synthesis or degradation of neurotransmitters. Thus, changes in basic cell functions *per se* cannot be used as indicators for neurotoxicity.

Some of the most common determinants of basic cell functions are listed in Appendix Table C-5.

C.3.5. Biochemical Parameters that can be measured in Body Fluids

In medical diagnostics many parameters measured in plasma, serum, or urine are used as indicators of organ dysfunction or toxicity. Such a diagnostic tool has the advantage to be minimally invasive and at the same time allows repeated determinations in the same individual.

Only a few parameters are known which identify neurotoxic effects. Inhibition of serum transketolase was shown to be accompanied by sensory loss and severe weakness in the legs. Activity of the enzyme returned to normal as clinical evidence of neuropathy disappeared (McVicar *et al*, 1973). Transketolase activity has however not been validated using other neurotoxicants.

Inhibition of blood acetylcholinesterase is partially correlated with clinical signs of intoxication induced by organophosphorous pesticides. Although this parameter cannot be used as a direct measure of central cholinergic intoxication, it gives a good indication for the exposure of individuals to these compounds (Gage, 1967).

The end products of neurotransmitter degradation can be measured in cerebrospinal fluid, blood or urine and have been used in man to confirm altered neurotransmitter functions related to psychiatric illness (Ackenheil, 1980; Langer and Karobath, 1980; van Praag, 1982; Dodd *et al*, 1988). Interpretation of such data is severely limited by the almost ubiquitous presence of many of the neurotransmitters in peripheral tissues and the altered elimination of these catabolites being secondary to impaired kidney function. These measurements cannot be used as indicators for potential neurotoxic effects.

C.3.6. Staining Techniques Adapted from Biochemical Methods

Most tissue sections are colourless when unstained. To increase distinction between individual cells or cell organelles different methods of staining have been introduced. Staining techniques make use of chemical or physical properties of dyes such as their preferential reactivity with acidic (haematoxylin, toluidine blue) or basic (eosin, acid fuchsin) groups or their high fat solubility (Sudan black).

The detection by Falck and Hillarp (1959) that formaldehyde reacts with catecholamines to produce fluorescent compounds led to visualisation of the catecholaminergic pathways in the brain (Ungerstedt, 1971). Fluorescence histochemistry has also been used in

neurotoxicity studies to assess lesions in monoaminergic systems (Krinke and Hess, 1981). Glutathione, a reducing molecule and important substrate in cellular defence mechanisms against oxidative stress, can be specifically stained with mercury orange or o-phthaldialdehyde. A reduction in concentrations of glutathione is indicative of an ongoing toxic process which may lead to a neurotoxic effect (Philbert *et al*, 1991).

2-Deoxy-D-glucose (2-DG) is a glucose analogue that, when taken up by cells, is phosphorylated but not further metabolised. As a phosphorylated sugar it is excreted only slowly and thus accumulates in cells. Intracellular concentration of 2-DG is related to functional activity. By using [^{14}C]2-DG the metabolic activity of specific brain areas can be estimated semi-quantitatively in autoradiographs of brain sections (Sokoloff, 1977). Neural activity is altered locally in brains bearing lesions induced by chemical or mechanical insult (Patel *et al*, 1974; Schwartz, 1978; Muller and Martin, 1984; Orzi *et al*, 1988). Similar changes in neural activity can also be induced by sensory stimulation (Sharp *et al*, 1975), alterations in activity, arousal or emotion (Roland *et al*, 1982; LeDoux *et al*, 1983), or by pharmacological stimulation (Delanoy and Dunn, 1978; Muller and Martin, 1984). Thus neurotoxic effects cannot be diagnosed based exclusively on alterations in the pattern of 2-DG accumulation. Rather it is only in the absence of any changes that a neurotoxic effect can be excluded.

Radioactively labelled molecules can also be used to assess neurotoxicant-induced changes in local receptor densities (Schlichter, 1979), protein synthesis, enzyme activity (Ogawa *et al*, 1989), neurotransmitter synthesis, cell proliferation (Miyake *et al*, 1989) or axonal transport rates (Monaco *et al*, 1985; Miller and Spencer, 1984).

The production of radiolabelled molecules and specific antibodies and their combination with histological techniques has opened a new era in histopathology. Antibodies against specific proteins can be coupled to fluorescent or electron-dense compounds or to enzymes that are directly visible in fluorescence or electron microscope or can be made visible by a chemical reaction (e.g., peroxidase reaction with 3,3'-diaminoazobenzidine). The sensitivity of this method can be increased using an indirect immunohistochemical method: specific proteins are localised with unmarked antibodies that are visualised in sections using marked antibodies binding to the long arm of the first antibodies. These specific staining techniques can demonstrate the distribution and local concentration of enzymes, neurotransmitters, receptors, cell-type specific marker molecules etc.

With the help of this new technology, many of the changes described in the above sections and originally measured in brain homogenates may now be investigated in brain sections together with their concomitant histological changes and may be traced back to

brain areas, cell groups or even single cells. Due to the increased topographic resolution, the accuracy of these staining methods is superior to that of the original biochemical techniques.

Such immunohistochemical techniques are increasingly used to detect or further characterise lesions induced by neurotoxicants (O'Callaghan, 1988; Streit and Kreuzberg, 1988; Miyake *et al*, 1989; Ogawa *et al*, 1989).

C.4. NEUROPATHOLOGICAL METHODS

C.4.1. Immersion Fixation for Neuropathology

The principles and techniques of immersion fixation of the nervous system in formalin with subsequent paraffin embedding are detailed elsewhere (AFIP, 1968; Ralis *et al*, 1973; WHO, 1986). Initial selection of sections for microscopic examination is based primarily on the knowledge that particular sites in the central and peripheral nervous system are most vulnerable to neurotoxic effects (Hirano and Llena, 1980; Thomas, 1980). Areas selected to give a thorough morphological assessment should thus include those listed in Appendix Table C-6. Haemotoxylin and eosin is the routine stain of choice and several special stains may be used to highlight pathological changes in components such as vasculature, cell bodies, axons and myelin (Appendix Table C-7).

Immersion fixation allows examination of large paraffin-fixed sections of nervous system in standard toxicology studies without the disadvantages of perfusion fixation (Appendix Table C-8). Resolution of cell detail in immersion-fixed paraffin sections is less than in plastic-embedded material and artefactual changes may occur (Cammermeyer, 1960; Garman, 1990). In the hands of the experienced neuropathologist this technique is a valuable first tier screen to detect the presence or absence of neuromorphological change.

C.4.2. Perfusion Fixation

Perfusion fixation is a useful procedure for specific investigations of neuropathological changes in the central or peripheral nervous system (Krinke, 1989; O'Donoghue, 1989; Mattsson *et al*, 1990b). Several authors have detailed the principles and techniques, based on intravascular perfusion of the whole body or CNS with isotonic, buffered solutions of saline, formalin, paraformaldehyde and/or glutaraldehyde (Zeman and Innes, 1963; Hayat, 1970; Palay and Chan-Palay, 1974; Spencer *et al*, 1980a; WHO, 1986). Perfused nervous tissue may subsequently be prepared for vibratome sections and histochemistry or immunocytochemistry (Berry *et al*, 1988), embedded in paraffin for

routine light microscopy or post-fixed in osmium tetroxide and processed for plastic-embedded semithin (1-2 micron) sections, usually stained with toluidine blue.

The main advantages and disadvantages of perfusion fixation are summarised in Appendix Table C-8. Central and peripheral nervous system tissue fixed by perfusion is devoid of most artefacts associated with immersion fixation (Garman, 1990). Thus, perfusion fixation may reduce false positive findings. Nevertheless, the perfusion procedure itself may be associated with particular artefacts, for example those caused by inadequate control of pressure, pH or osmolarity of the infused fixative (Schultz and Karlsson, 1965). Fine cellular detail, including organellae such as mitochondria, is normally discernable by light microscopic examination of well perfused tissue, especially in semithin (1-2 micron) plastic-embedded sections post-fixed in osmium tetroxide. This fixative preserves lipids lost by routine processing for paraffin sections. Perfusion fixation has been used to define the neurotoxic effect of chemicals in a number of species including rats (Cavanagh, 1973; Spencer and Schaumburg, 1975; Brzoska and Adhami, 1976; Burek *et al*, 1980; Jones and Cavanagh, 1981), rabbits (Render and Carlton, 1985; Triarhou *et al*, 1985; Anderson and Davidovich, 1990), guinea pigs (Parhad *et al*, 1982; Finnie and O'Shea, 1988), dogs (Krinke *et al*, 1979; Parhad *et al*, 1982; Schaeppi *et al*, 1984; Berry *et al*, 1988) and non-human primates (Baumbach *et al*, 1977; Thomas *et al*, 1984; Eskin *et al*, 1985).

C.4.3. Teased Nerve Fibre Preparations

The technique of nerve fibre teasing involves separation under a dissecting microscope of individual perfusion-fixed peripheral nerve fibres embedded in epoxy resin of low viscosity (Dyck and Lais, 1970; Spencer and Thomas, 1970). Each separated fibre, comprising an axon and its myelin sheath, can be examined microscopically using bright-field or Normarski optics. Used in conjunction with plastic-embedded semi-thin sections, morphometry, immunohisto- chemistry or electron microscopy nerve fibre teasing may provide a valuable means to study axonal and myelin degeneration, remyelination and other pathogenetic changes in peripheral neuropathy (Spencer *et al*, 1980a; Griffin, 1990). However, the principal disadvantage of the method is its laborious and time consuming nature (Appendix Table C-8). Nerve fibre teasing is therefore best confined to investigations specifically aimed at detailed study of the morphogenesis of chemically-induced peripheral neuropathies, such as those reported with hexacarbon compounds, acrylamide, clioquinol, trimethylphosphate and pyridoxine (Spencer and Schaumburg, 1976; Krinke *et al*, 1979; Schaeppi *et al*, 1984; Krinke *et al*, 1985). Teased-fiber preparations can be used on a limited number of selected specimens to discriminate between primary axonal lesions and primary segmental demyelination.

C.4.4. Transmission Electron Microscopy

The techniques used in the preparation of ultrathin sections of central and peripheral nervous system for transmission electron microscopy are described in the literature and several standard texts (Hayat, 1970; Palay and Chan-Palay, 1974; Spencer *et al*, 1980a). In summary, small blocks of nervous tissue preserved by perfusion in a glutaraldehyde-based fixative are post-fixed in osmium tetroxide and embedded in plastic (epoxy resin). Light microscopic examination of semithin (1-2 micron) toluidine blue stained sections is used to select areas of tissue for ultrastructural study. Ultrathin (approximately 50nm) sections cut usually with a diamond knife are impregnated with heavy metal stains such as lead citrate and uranyl citrate are examined and photographed in a transmission electron microscope.

The decision to use electron microscopy must be carefully considered in testing chemicals for neurotoxicity. The ultrastructural identification of organellar targets of neurotoxic chemicals (Hirano and Llena, 1980; Thomas, 1980; Price and Griffin, 1980; WHO, 1986) may provide a basis for the formulation of hypotheses concerning their mechanism of action. Results of electron microscopic studies can also support regulatory submissions for pharmaceuticals and other chemicals. For example, in the preclinical development of the new cholesterol-lowering drug lovastatin, ultrastructural studies demonstrated a safety advantage of the new compound by showing an absence of CNS intracellular membranous inclusions typically associated with other inhibitors of cholesterol synthesis such as triparanol and AY9944 (Suzuki and Zagoren, 1974; Berry *et al*, 1988)

Although electron microscopy is a powerful tool in mechanistic studies of neurotoxicology, its application is laborious and requires extensive training and experience (WHO, 1986). In testing chemicals for neurotoxicity, ultrastructural studies should therefore be confined to those protocols where there is a specific need to answer mechanistic questions and more clearly define ultrastructural effects.

C.4.5. Morphological Methods Based on Immunohistochemistry

Principles of morphological staining techniques adapted from biochemical and immunological analytical methods are summarised in section C.3.6. There are numerous peptides in the nervous system which may potentially be demonstrated by immunohistochemistry during neurotoxicological processes. Some are characteristic of particular neural cell types (Raff *et al*, 1979; Schachner, 1982; De Blas *et al*, 1984). Others such as the 'heat shock' protein ubiquitin are expressed during pathological processes in a variety of cells including those of the central nervous system (Mayer, 1987;

Lowe *et al*, 1988). Certain laboratories have used batteries of selected neurotypic or gliotypic proteins in immunohistochemistry and tissue radio-immunoassay to test for pathological effects induced in the developing and mature central nervous system by a limited number of neurotoxic chemicals, particularly trimethyl- and triethyltin (Brock and O'Callaghan, 1987; O'Callaghan and Miller, 1988). Immunohistochemical quantification of activated macrophages and Schwann cell expression of nerve growth factor have also been proposed as screening tools for chemical-induced axonal degeneration and demyelination in the central or peripheral nervous system (Griffin, 1990).

Immunohistochemical methods have yet to be validated for general use in testing chemicals for neurotoxicity. The techniques have proved valuable research tools and certain procedures, such as that developed by O'Callaghan and Miller (1988) to demonstrate GFAP (glial fibrillary acidic protein), also show potential for use in neurotoxicity screening. Nevertheless, before the widespread use of immunohistochemistry in testing of new chemicals for neurotoxicity is introduced, the methods must be validated using a large number of neurotoxicants with a variety of morphological effects.

C.4.6. Morphometric Methods

Pathologists routinely use semiquantitative evaluations or grading of morphologic changes in tissues, including nervous tissues, from standard toxicological studies. Adequate definition of certain neuropathologic changes, such as particular effects induced during development (Rodier, 1990) or effects in the peripheral nervous system of adults (Dayan, 1979; Haug, 1986) may require quantitative methods including image analysis (Marchevsky *et al*, 1987).

The application of morphometric and stereologic methods to evaluate neuropathological changes have been reviewed by several investigators (Diemer, 1982; Broxup *et al*, 1989b, 1990; Korbo *et al*, 1990). Morphometric methods for the nervous system may be applied to images of different scale, ranging from sections of whole brain to highly magnified electron micrography of subcellular organellae such as synaptic vesicles. The considerations in sampling technique, preparation of specimens as well as the images to be analysed, are generally those applicable to morphometric assessment of all tissues.

The orderly arrangement of peripheral nerves facilitates quantitative morphometric evaluations. Changes in diameter of nerves may indicate atrophy, hypertrophy or swelling; changes in total number of nerve fibers reflect degeneration or propagation (after regeneration). Measurement of cellularity in peripheral nerves (e.g., by simply counting

cell nuclei on haematoxylin-eosin stained slides regardless of the cell type) can give a first indication for a degenerative process (Abercombie and Johnson, 1946). The distribution of fiber diameters, their form (circularity), the relation of axon diameter to myelin thickness or the density of certain organelles such as neurofilaments may allow more specific diagnosis of ongoing degenerative changes (Dyck *et al*, 1984).

Morphometric evaluation of the central nervous system has to take into account topography and structural features of the anatomic area as well as the specific effects induced by the compound (Diemer, 1982). Because of the anisotropic distribution of neurons and other functional units in the nervous system, morphometric methods should be selected on a case by case basis after special consideration of the anatomical location and character of the lesion.

C.5. DEVELOPMENTAL NEUROTOXICITY TESTS

The nervous system of newborn animals is not fully developed and thus can support only part of the functions of an adult animals' nervous system; the functional status of the nervous system of pups requires different test methods. On one hand, the continuing development of the nervous system of pups result in increasing functional capabilities. On the other hand, the developing nervous system can more readily adapt to or compensate for functional losses as the result of a toxic insult. Even when functional losses can be fully compensated over time they result in a delayed functional development. The age at which a certain function is fully developed is a sensitive parameter indicating toxic effects on the developing nervous system. In the following section, some of the methods that are frequently used for the detection of developmental neurotoxicity are described. For more tests and methods the reader is referred to Altman and Sudarshan (1975), Adams (1986), WHO (1986) and Appendix Table C-9.

C.5.1. Functional Landmarks

Surface righting. Animals are placed in a supine position and the time required to turn over onto the legs is measured. This reflex is present soon after birth, but the time required for the animals to turn decreases with age (Altman and Sudarshan, 1975).

Negative geotaxis. When rats are placed head down on an inclined plane the normal reflex is to turn to face upward. This test can be performed with rats from post-natal day 5 onwards; the speed of turning increases rapidly and asymptotic latency is reached by day 8 (Altman and Sudarshan, 1975).

Pivoting. Pivoting is a circular locomotor movement of pups resulting from the fact, that in 4 to 5-day old rats the forelimb function is better developed than hind-limb function (Altman and Sudarshan, 1975). As soon as the animals can coordinate fore and hind-limb function, head and pelvis, pivoting disappears and they start walking on all 4 limbs (usually from day 6 onwards).

Mid-air righting. A reflex similar to surface righting is the mid-air righting; the animals are dropped from about 60 cm height and should land on their limbs. This ability develops between 12 and 17 days of age in Wistar rats (Altman and Sudarshan, 1975).

Swimming ontogeny. Swimming is a complex coordinated movement which develops between 6 and 25 days after birth. This development of swimming ability can be scored with respect to body position and limb usage (Schapiro *et al*, 1970).

Crossing paths of different widths. In order to reach their home cage, developing rats are required to traverse paths of different lengths and widths. This test measures the development of certain locomotor abilities when balancing is required and is usually conducted from day 12 onwards (Adams, 1986).

Rotarod. Motor coordination and balancing abilities can be determined using a rotating rod (Altman and Sudarshan, 1975). Varying speed or diameter of the rod may increase the information obtained from this test. This test is usually conducted from day 18 onwards (Adams, 1986).

Hanging grip strength. The forelimbs of the animal are placed on a thin wire and the ability and/or duration of grasping and holding is measured. From 13 days of age onwards, the latency to fall is measured within a 1-minute test period (Adams, 1986).

Hind-limb support when suspended. Suspended rats tend to grasp a horizontally extended string or wire. This is followed by attempts at pull-ups with the forelimbs and the synergistic support of the body with the hind-limbs. The test can be performed from day 11 onwards, but the animals do not respond consistently until day 21 (Altman and Sudarshan, 1975).

Jumping down to home cage. This test measures the latency required for a rat to jump down from a platform into the home cage. The platform is placed between the home cage and an empty cage, therefore also the direction of the jump can be recorded. More or less consistent downward jumping from heights of 35 and 50 cm is not apparent until day 12 (Altman and Sudarshan, 1975).

C.5.2. Specific Tests

Locomotor activity. Locomotor activity of developing animals can be determined in an open field or a maze as described for adult animals (cf. C.1.2). In the rat, a peak of motor activity usually occurs between 14 and 18 days of age and falls to adult levels at about four weeks of age (WHO, 1986). As already mentioned for adult animals, motor activity alone is not a specific indicator in developing animals for neurotoxicity and the results obtained from these measurements must always be assessed in the context of systemic toxicity.

Sensory functions. Tests to assess sensory function are based on the response to certain stimuli. As they rely on indirect measurements, they can be influenced by a wide range of other variables (Evans, 1978).

Auditory startle reflex. The response of animals to a sudden noise (i.e., a finger snap, or an electrically produced sound) is observed. A normal reaction of the animal is movement of the ears or a jerk. The response develops in rats approximately on postnatal day 12 (Vorhees *et al*, 1979).

Olfactory orientation. Nest-seeking behaviour can be used in assessing the ability of young rats to discriminate between the odour of the home cage (soiled home-bedding material) and a novel cage (clean bedding). The pups are placed in between soiled bedding and clean bedding, and the directional orientation to the home odour or the time to reach the home bedding is measured (Altman and Sudarshan, 1975). The ability develops in rats on approximately postnatal day 8 (Gregory and Pfaff, 1971).

Learning ability. The experimental procedures for learning ability can be divided in habituation, classical conditioning, and operant behaviour. For these tests usually older animals are used and the methods are described in C.1.4.

C.6. ALTERNATIVE *IN VIVO* ASSAYS: LOWER VERTEBRATE MODELS (cf. Appendix Table C-10)

Lower vertebrate models may be employed to investigate neurotoxic mechanisms since the level of cellular and tissue organisation exceeds that found in tissue cultures.

The chick embryo represents such an alternative *in vivo* assay (Hirano and Kochem, 1973). Compounds are administered in precisely controlled doses to 4-day-old chick embryos and the effects on developing processes of the nervous system can be investigated since the stages of differentiation are well defined. The nervous system is visible early in development

and is relatively easy to fix for light and electron microscopic studies. A further advantage is the ability to maintain large groups of test and control embryos.

Another potentially useful *in vivo* model is the *Xenopus* tadpole (Webster *et al*, 1980). Dose-dependent effects of compounds, injected subcutaneously or added to the culture medium, on the differentiation and development of the nervous system or on the outgrowth and myelination of nerve fibres in the tailfins or of the optic nerves can be observed. Similarities between fine structure of the tadpole and developing rodent neural cells and the ability to correlate behavioural responses with the distribution of lesions give some advantages.

Irrespective of the usual disadvantages (absence of a blood brain barrier, difficulties in interpretation and definition of endpoints) the main limitation of both models is a lack of detailed validation studies with a sufficient number of neurotoxins in different laboratories. The chick embryo model has been used to examine the neurotoxic effect of lead (Hirano and Kochen, 1973) whereas myelin lesions produced by hexachlorophene and by the cerebrospinal fluid from multiple sclerosis patients have been investigated in the *Xenopus* tadpole model (Tabira *et al*, 1977).

C.7. ALTERNATIVE *IN VITRO* ASSAYS (cf. Appendix Table C-11)

C.7.1. Membrane Models

Membrane models such as isolated human erythrocyte membranes and rat cerebral synaptosome membranes represent *in vitro* methods for detecting membrane effects as neurotoxic mechanisms. Cell membrane changes are studied by measuring changes in the activity of the integral cell membrane enzymes acetylcholinesterase (AChE) and adenosinetriphosphatase (ATPase). The acute anaesthetic effects of organic solvents may be mediated by changes in neural membrane integral proteins and this effect can be investigated in many cell-types, including peripheral non-excitable cells such as erythrocytes. A number of organic solvents including toluene, carbon disulphide, n-hexane and its metabolites 2-hexanone and 2,5-hexanedione have been investigated (Taehti and Hyppoenen, 1990).

C.7.2. Primary Neuronal Cultures

Neural tissue from selected regions of the immature rat nervous system can be mechanically and enzymatically dissociated to produce a mixture of individual neurons and glial cells. Neurons are non-dividing cells, so only primary cultures are possible. Primary neuronal cultures are useful in studies of membrane neurophysiology. Whole-cell voltage clamp, patch clamp, and current clamp studies can reveal toxicant-induced dysfunction of

voltage-gated and ligand-gated ion channels in a very direct way. These methods are useful in the study of compounds that interfere with normal operation of ion channels. The reversibility of the dysfunction can be studied by simply changing the bathing solutions. Primary cultures of central nervous system (CNS) and peripheral nervous system (PNS) neurons may be used and the choice of brain region depends on the particular experimental question.

Further advantages to primary neuronal cultures include the facts that the cells are real neurons rather than hybrid cells, that an individual neuron can be studied without the confounding influence of other neurons, and that chemically-induced changes in neuronal survival, neuronal morphology and dendritic growth can be determined. There are certain disadvantages such as the difficulty of maintaining the cells; the physiological properties of the immature neurons are sometimes different from their adult counterpart, e.g., voltage-dependent calcium channels have different activation voltages and inactivation kinetics, and that the neurons are deprived of their normal afferent and efferent targets.

Primary neuronal cultures probably have primary use in mechanistic analysis of known neurotoxicants. The information gained by mechanistic studies with primary neuronal cultures have to be verified *in vivo* to ensure that the mechanisms are not peculiar to the unnatural setting of immature neurons in a dish.

The electrophysiology of excitotoxic compounds which interact with subsets of glutamate receptors have been studied in detail with primary cultures of hippocampal (Mayer and Westbrook, 1987) and neocortical neurons (Choi *et al*, 1988). Choi (1990) has reviewed a number of physiological and biochemical events such as elevation in intracellular calcium or activation of proteolytic enzymes that occur during the process of ischemia-induced neuronal death, and measurement of these events could prove to be useful endpoints in neurotoxicology. Further examples of studies involving primary neuronal cultures include the investigation of the effects of acrylamide (Hooisma, 1982), lead acetate (Holtzman *et al*, 1987), and methylated halogens (Davenport *et al*, 1989) on the morphology of cultured cerebellar neurons.

C.7.3. Glial Cell Cultures

Glial cells perform numerous functions in the nervous system. They support neurons physically, biochemically, and electrically. Neuronal damage triggers glial cell responses, and glia are themselves direct targets for chemically-induced damage. Glial cells are dividing cells and several successive generations of glial cells can be maintained in culture.

Schwann cells are glia that produce the myelin sheath which electrically insulates axons in the peripheral nervous system. They can be harvested from the peripheral nerve bundles of immature and adult animals and even post-mortem human beings (Morrissey *et al*, 1990) and maintained in culture (Assouline *et al*, 1989). These cells can be triggered to produce myelin by adding neurons to the culture, or by adding components of the neuronal membrane (Porter *et al*, 1986).

Schwann cell cultures may be a promising tool in neurotoxicology because there are chemicals that specifically attack the Schwann cell or the myelin they produce, or both. For example triethyltin and lysolecithin cause demyelinating disease without damaging the myelinating cells whereas lead and carbon monoxide injure both myelin and myelinating cells (Cammer, 1980). Mithen *et al* (1990) have used myelin-forming cultures of Schwann cells to examine ethanol toxicity. Schwann cells in culture can be an appropriate model for screening new compounds for their demyelinating potential. Primary cultures of rat and mouse astrocytes have also been used to investigate the species-specific toxic effects of α-chlorhydrin and 6-chloro-6-deoxyglucose and to confirm the effects observed *in vivo* (Evans and Fawthrop, 1990).

C.7.4. Cell Lines

Clonal cell lines may be useful in understanding the cell biology of neurotoxic agents. Examples are the PC-12 cell line derived from a pheochromocytoma of the rat adrenal medulla, and neuroblastoma/glioma hybrid lines such as NG 108-15. Those cell lines are model systems that share certain features with real neurons or glia. Cultured in the presence of nerve growth factor (NGF), PC-12 cells differentiate to resemble morphologically and functionally, sympathetic neurons. Thus, these cells provide a good model for studying the processes associated with function and regulation of neurotransmitters, ion channels and membrane bound receptors and the ability of compounds to interact with them. PC-12 cells have been used in studies of methylmercury and have shown compound-induced abnormalities in voltage-dependent calcium channel function (Atchison and Shafer, 1990).

Neuroblastoma/glioma hybrid cells have been employed to study the effects of lead ions on calcium channel function (Audesirk and Audesirk, 1990). Cell lines have also been used to examine the neurotoxic effects of organophosphorous compounds (Carrington *et al*, 1985), pyrethroid insecticides (Rowan, 1985; Oortgiesen *et al*, 1989), various industrial solvents (Selkoe *et al*, 1978), and acrylamide (Walum *et al*, 1987).

C.7.5. Organotypic Explants

An organotypic explant is a piece of tissue that retains some of the structural and functional characteristics typical of the organ from which it was taken (Peterson *et al*, 1965). Explants from the brain have to be removed from embryonic animals to enable survival of the explants for weeks or months.

Disadvantages to the use of organic explants are similar to those of primary neuronal cultures. In addition, preparing and maintaining explants is tedious and time-consuming. Because the neurons in the explant are immature, the relevance of experimental results to the mature nervous system is doubtful. Furthermore, while the explant can be demonstrated to develop many kinds of normal synaptic contact, it is disconnected from many of its normal afferents, and this could be relevant if the *in vivo* effects of a test agent are on a brain region that normally sends efferents to the explanted tissue. As with primary neuronal cultures, the use of organotypic explants is best limited to use in mechanistic studies where it is already known that the chemical has an adverse effect on a type of neuron within the explanted region or affects the general process of axonal growth and synapse formation.

Yonezawa *et al* (1980) reviewed some applications of organotypic cultures for the study of a number of chemicals including methyl mercury, thallium, tellurium, triethyltin, clioquinol, chloroquine, 2,5-hexanediol.

Co-cultures of spinal cord and muscle offer opportunities to examine the effect of chemicals on development of the neuromuscular junction. Such preparations have been used to examine hexacarbon solvents (Veronesi *et al*, 1984) and organophosphates (Tuler and Bowen, 1989). Most of the neurotoxicological work has used morphological endpoints, but biochemical and electrophysiological measurements are also possible.

C.7.6. Rotation-mediated Aggregating Cultures

This organotypic culture system consists of whole or part brain enzymatically or mechanically dissociated into single cells. The cell suspensions are inoculated into constantly rotating flasks where the cells reaggregate into clusters that exist in suspension due to the vortex of rotation. The cells interact by cell-cell contacts in three dimensions and organise themselves into aggregates which architecturally resemble the brain area sampled. These aggregates can be sampled at various stages as the cells develop from undifferentiated neuroepithelial cells to a population of morphologically mature neurons, astrocytes and oligodendrocytes.

The main advantage of reaggregates is their ease of preparation, reproducibility and representation of the whole-brain response rather than just one individual area or cell type. The biochemical characterisation of aggregating cell cultures exceeds that of any primary CNS culture system, thus offering a distinct advantage for multidisciplinary investigation of the CNS *in vitro*.

However, it is impossible to perform routine electrophysiological examination as the tissue exists in suspension. Another problem is the extrapolation to the mature CNS because the reaggregates predominantly show the profile of neurotoxicity in the developing CNS. Furthermore, a large number of foetuses has to be used.

For many neurotoxicants a correlation between their known *in vivo* effects and functional perturbations produced in developing organotypic brain reaggregate cultures *in vitro* has been found. These include methylmercury, cycloheximide and colchicine (Jacobs *et al*, 1986), 6-hydroxydopamine and the tricyclic antidepressants (Majocha *et al*, 1981), ascorbic acid and kainic acid (Trapp and Richelson, 1980), organophosphorous compounds (Wehner *et al*, 1985), ethylcholine mustard aziridinium and aluminium (Atterwill and Collins, 1988; Atterwill, 1990).

TABLE C-1 Examples of Behavioural Procedures included in a Functional Observational Battery

Home-cage and open field	Manipulative	Physiological
posture	ease of removal	body temperature
convulsions	ease of handling	body weight
palpebral closure	palpebral closure	
lacrimation	approach response	
piloerection	touch response	
salivation	finger-snap response	
vocalisations	tail-pinch response	
time to first step	toe pinch response	
tremors	pupil response	
rearing	pineal response	
stereotypy	eye blink reflex	
gait	righting reflex	
urination	catalepsy	
defecation	hind-limb foot splay	
arousal	forelimb grip strength	
respiration	hind-limb grip strength	
	positive geotropism	
	wire manoeuvre	

TABLE C-2 Behavioural Tests

Test	Endpoint Parameter(s)	(+) Advantages and (-) Disadvantages	Species	Effective Substance	References
Rotating rods (rotarod, accelerod)	motor function	(-) training required (-) rather intensive (-) great variance of data (depends on willingness and ability of the animal)	rat mouse	acrylamide, ethanol	Bogo *et al*, 1981 Kaplan and Murphy, 1972
Inclined planes	motor function	(+) no training required (-) rather intensive	rat	methylmercury	Fehling *et al*, 1975
Landing footspread (hindlimb foot splay)	motor function	(+) no training required (-) insufficiently sensitive	rat	acrylamide chloridimeform, 3-acetyl pyridine	Edwards and Parker, 1977 Broxup *et al*, 1989a Moser *et al*, 1988 Moser and MacPhail, 1990
Swimming ability	motor function	(+) sensitive ( ) observer bias	mouse	methylmercury	Spyker *et al*, 1972
Fore and hindlimb grip strength	motor function	(+) sufficiently sensitive for screening (+) reproducable (-) may be confounded by other factors like habituation, toe-nail injuries, general sickness	rat mouse	chlordiazepoxide, phenolbarbital acrylamide, chlordimeform carbyl, 3-acetyl pyridine tetraethyltin, methylmercury, monosodium-salicylate	Meyer *et al*, 1979 Broxup *et al*, 1989 Moser *et al*, 1988 Moser and MacPhail, 1990 Pryor *et al*, 1983
Mazes (figure-8, residential)	motor activity	(+) no training required (-) insensitive	rat	trimethyltin acrylamide, ethanol, amphetamine lead	Ruppert *et al*, 1982 Broxup *et al*, 1989 Reiter, 1978
Open field	motor activity	(-) insensitive	rat	amphetamine	Schiorring, 1979

TABLE C-2 Behavioural Tests (continued)

Test	Endpoint Parameter(s)	(+) Advantages and (-) Disadvantages	Species	Effective Substance	Reference
T-maze, Radial arm maze, Water maze	spatial discrimination, learning, short and long term-memory	(+) specific deficits in cognitive function can be analysed (-) dependent on intact sensometric function (-) training sometimes required	rat	AF64A organophosphates trimethyltin, AF54A kainic acid N-methyl-D-aspartate (NMDA)	Chrobak *et al*, 1987 McDonald *et al*, 1988 Walsh and Chrobak, 1987 Milgram *et al*, 1988 Rogers and Tilson, 1990
Visual discrimination learning/ Discrimination reversal	nonspatial discrimination learning/reversal	(+) specific deficits in cognitive function can be detected (-) training required	rat monkey	lead lead, cadmium lead	Winneke *et al*, 1977 Winneke *et al*, 1986 Rice, 1985
Delayed matching to sample	concept formation and short-term memory	(+) specific deficits in cognitive function can be detected (-) training required	pigeon monkey	pentobarbital, phencyclidine lead	McMillan, 1981 Rice, 1984
Schedule controlled operant behaviour	cognitive function measured by a fixed rate (FR) or fixed interval (FI) schedule	(+) generality across species (-) dependent on intact sensometric function (-) food or water deprivation required (-) training required (-) not specific for neurotoxic compunds	rat	lead methyl n-amyl ketone acrylamide chlordimeform ozone chlordimeform, carbaryl, triadimefon, nicotine, pentobarbital, 3-acetylpyridine	Dietz *et al*, 1978 Cory-Slechta *et al*, 1981 Anger *et al*, 1979 Tilson *et al*, 1980 MacPhail and Leander, 1981 Weiss *et al*, 1981 Moser and MacPhail, 1990

TABLE C-2 Behavioural Tests (continued)

Test	Endpoint Parameter(s)	(+) Advantages and (-) Disadvantages	Species	Effective Substance	Refernces
Modulation of reflex startle	acoustic function	(+) sensitive by use of low-intensity stimuli	rat	acrylamide, chlordecone, tetraethyltin, triethyltin	Pryor *et al*, 1983 Fechter and Young, 1983
Auditory thresholds	acoustic function	(+) appropriate for specific studies (-) time consuming	monkey	aminoglycosidic antibiotics	Stebbins and Rudy, 1978
Visual discrimination	visual function	(+) appropriate for specific studies	pigeon, rat, monkey	LSD LSD acrylamide	Blough, 1957 Friedman and Carey, 1978 Merigan *et al*, 1982
Assessment of spatial vision	visual function	(+) appropriate for specific studies	monkey	methylmercury	Rice and Gilbert, 1982
Somatosensory thresholds	sensory function, vibratory and electrical sensitivity	(+) appropriate for investigating peripheral neuropathies	monkey	acrylamide	Maurissen *et al*, 1983
Thermal sensitivity	pain test, tailflick latency	(+) routine test in pharmacology	rat	triethyllead	Pryor *et al*, 1983
Multisensory conditioned avoidance response (CAR)	acoustic, visual and sensory function	(+) simultaneous measurement of three modalities	rat	acrylamide, chlordecone, methylmercury, tetraethyltin	Pryor *et al*, 1983
Photocell cages	motor activity	(-) insensitive (-) undifferentiated	rat	arsenic, lead acetate, tetraethyltin	Pryor *et al*, 1983
Flavour-aversion conditioning	associative learning	(+) independent on motoric competence (-) interpretation is difficult (-) not specific for neurotoxic effects	rat	triethyltin, trimethyltin, 3,3'-iminodiproprionitrile IDPN	MacPahil, 1982 Peele *et al*, 1990
Active and passive avoidance	learning and memory	(+) no food and water deprivation required (+) no training required (-) dependent on sensometric competance	rat	lead IDPN	Sobotka *et al*, 1975 Peele *et al*, 1980

TABLE C-3 Neurotoxic Mechanisms and Related Chemicals (adapted from Spencer and Schaumburg, 1980b)

Probable Neurotoxic Mechanism	Chemical
stimulation of excitatory receptors	NMDA, kainate, quisqualate
neurotransmitter metabolism	hemicholinium, reserpine
release of neurotransmitters	tetanustoxin, botulinustoxin
membrane permeability	hexachlorophene, triethyltin
Na-, K- and Ca-channels	p,p'-DDT, chlordecone
axoplasmatic flow	hexanedione, iminoproprionitrile, acrylamide
energy metabolism	cyanide, carbon monoxide, 6-chloro-6-deoxyglucose, 6-amino-nicotinamide
energy supply	histamine, arachidonic acid, cyanide
protein synthesis	acrylamide, doxorubicin, aluminium
lipid synthesis	tellurium
catabolism	chlorophentermine, chloroquine

TABLE C-4 Most Common Cell-type Specific Marker Molecules, Corresponding Cell Type and Their Subcellular Localisation

CELL TYPE	SUBCELLULAR DISTRIBUTION	MARKER MOLECULE
Neurons	synapses	gangliosides, synapsin I
	synaptic vesicles	neurotransmitters, choline acetyltransferase
	intermediate filaments	neuronal intermediate filaments
	microtubules	microtubule associated protein (MAP-2)
	membrane	neuronal cell adhesion molecule receptors
	cytoplasm	tyrosine hydroxylase, glutamate decarboxylase, glutamine synthetase, dopamine-β-hydroxylase etc., neuron specific or γ-enolase
Glia cells	intermediate filaments	glial fibrillary acidic protein (GFAP) S-100 protein
	myelin	myelin basic protein (MBP)
	cytoplasm	glutamate synthetase, 2',3'-cyclic nucleotide-3'-phospho-hydrolase non-neuronal or α-enolase creatine kinase-B transketolase
	membrane	cerebrosides, sulphatides
	myelin	P_o-protein, cholesterol
Macrophages	lysosomes	β-glucuronidase β-galactosidase MHC II

TABLE C-5 Some of the Most Common Determinants of Basic Cell Functions

CELL FUNCTION	PARAMETER
Cell viability	exclusion of trypan blue uptake of neutral red or kenacid blue
Membrane permeability and function	intracellular Ca^{++}-concentration excretion of intracellular enzymes (eg. LDH) Na-K/Ca-Mg-ATPase activity
Energy metabolism	glucose utilisation (eg. 2-DG uptake) concentration of ATP activity of succinate-dehydrogenase activity of 6-phosphate-dehydrogenase
RNA Synthesis	incorporation of radiolabelled nucleic acids
Protein Synthesis	incorporation of radiolabelled amino acids
Cell proliferation	ornithine decarboxylase
Cell protection	concentration of glutathione catalase, superoxide dismutes etc., production of malondialdehyde
Axonal transport	transport of radiolabelled protein, gangliosides etc.

TABLE C-6 Regions of Nervous System Recommended for Microscopic Examination in Tier 2 Studies

Region of nervous system	Section/s recommended
Central nervous system	Forebrain (with basal ganglia) Centre of cerebrum Hippocampus Midbrain Cerebellum and pons Medulla oblongata Spinal cord (cervical, lumbar enlargements)
Peripheral nervous system	Dorsal root ganglia (cervical, lumbar) Peripheral nerve (sciatic, tibial) Eye (longditudonal section including retina, optic nerve and papilla)

TABLE C-7 Special Stains for Use in Formalin-fixed Paraffin-embedded Central and Peripheral Nervous System

Tissue component demonstrated	Special stain method	Reference
Basement membranes, extravasated glyco-protein, fibrinoid	Periodic acid-Schiff	AFIP (1968) Ralis *et al* (1973)
Neuronal (and astrocytic) cell bodies	Einarson's gallocyanin, Cresyl echt violet	AFIP (1968) Ralis *et al* (1973)
Axons	Bodian's silver, Sevier-Munger's silver	AFIP (1968) Ralis *et al* (1973)
Myelin	Kluver's luxol fast blue	AFIP (1968) Ralis *et al* (1973)
Degenerated myelin	Marchi's stain	Swank and Davenport (1934, 1935)
Neurofibrillary components of axons	Belckosky's silver, Glees and Marsland	AFIP (1968) Ralis *et al* (1973) Marsland *et al* (1954)

TABLE C-8 Comparison of Pathological Techniques for Light Microscopic Examination of Neurotoxic Changes in the Central and Peripheral Nervous System

Pathological technique	Advantages	Disadvantages
Immersion-fixed paraffin sections	Rapid, least expensive, can survey large areas of routinely-stained tissues in 'stage 1' screen. Can be used with standard toxicology protocols. Will demonstrate most disease processes in high dosed animals. Additional special stains applicable. Immunohistochemistry applicable.	Artefactual changes may lead to false positives. Cellular resolution less detailed. Some disease processes not recognisable. Artefactual translocation of immunogenic determinants in ICC.
Immersion-fixed plastic sections	Increased cellular detail may aid interpretation.	Increased detail may be offset by artefactual changes. Section size may be limited unless particular techniques used. Limited number of staining techniques applicable.

TABLE C-8 (continued)

Pathological technique	Advantages	Disadvantages
Perfusion-fixed paraffin sections	Virtual absence of artefact in well prepared tissue. Large sections can be prepared for examination. Most morphological changes easily recognised. Special stains readily applicable with formalin perfusion and ICC is possible.	Slow, time consuming. Cannot be used on standard toxicology studies; additional animals must be allocated to allow gross examination and organ weighing. Special stains less applicable with glutaraldehyde fixation. Glutaraldehyde vapours are irritant.
Perfusion-fixed plastic sections.	Excellent visualisation of cellular detail. Virtual absence of artefact in well prepared tissue. Can be readily combined with electron microscopy to study pathenogenesis.	Slower preparation than paraffin-embedded sections. Only comparatively small sections can be easily prepared unless particular techniques used. Limited number of staining techniques applicable.
Perfusion-fixed plastic teased nerve fibre preparations.	For characterisation of changes excellent demonstration of ongoing Wallerian-like degeneration, segmental demyelination and changes in internode length in remyelinated and regenerated fibres, Waller (1852)	Less amenable to immunohistochemistry. Laborious, time consuming.

Adapted from Griffin (1990)

Table C-9 Developmental Neurotoxicity Screening

Test	Endpoint Parameter(s)	Age at testing	Reference
Surface righting	motor function	3 days	Adams, 1986
Pivoting	motor function	4 days	Altman and Sudarshan, 1975
Cliff avoidance	motor function, sensory function	5 days	Altman and Sudarshan, 1975
Swimming ontogeny	motor function	6 days	Schapiro *et al*, 1970
Negative geotaxis	motor function	7 days	Altman and Sudarshan, 1975
Jumping down to home cage	motor function, sensory function	10 days	Adams, 1986
Mid-air righting	motor function	17 days	Altman and Sudarshan, 1975
Hindlimb support	motor function	12 days	Grauwiler and Leist, 1977
Grip strength	motor function	13 days	Altman and Sudarshan, 1975
Rotarod	motor function	18 days	Altman and Sudarshan, 1975
Neonatal T-maze	motor function	15 days	Adams, 1986
Figure-8 activity	motor function	15 days	Adams, 1986
Water maze	motor function	43 days	Adams, 1986
Ascending on wire mesh surface	motor function	8 days	Altman and Sudarshan, 1975
Olfactory orientation (nest seeking)	sensory function	8 days	Gregory and Pfaff, 1971
Auditory startle reflex	sensory function	12 days	Vorhees *et al*, 1979
Odor aversion	learning and memory	in the suckling period	Rudy and Cheatle, 1979
Taste aversion	learning and memory	in the suckling period	Adams, 1986
Passive avoidance	learning and memory	50 days	Adams, 1986
Active avoidance	learning and avoidance	after weaning	Adams, 1986

Table C-10 Alternative *in vivo* Assays

Test System	Endpoint Parameter(s)	(+) Advantages and (-) Disadvantages	Effective Substance	References
Chick embryo	mainly developmental effects of the nervous system (NS)	(+) well defined stages of differentiation (+) large groups of test control animals	lead salts	Hirano and Kochen, 1973
Xenopus tadpole	effects on development of the NS on outgrowth and myelination of nerve fibres and on behaviour	(+) level of organisation exceeds that found in tissue cultures (-) lack of detailed validation studies (-) interpretation on definition of endpoints is difficult (-) metabolism is differant to mammals	hexachlorophene, cerebrospinal fluid from multiple sclerosis patients	Tabira *et al*, 1977 Webster *et al*, 1980

Table C-11 *In vitro* Assays

Test System	Endpoint Parameter(s)	(+) Advantages and (-) Disadvantages	Effective Substance	References
Membrane models (erythrocyte and synaptosome membranes)	effects on integral cell membrance enzymes (AcHE, ATPase)	(+) useful for mechanistic studies (-) limited specifically to compounds which effect cell membranes	carbon disulphide, n-hexane and metabolites, toluene	Taehti and Hyppoenen, 1990
Primary neuronal cultures	effects on ion channels and on neuronal survival, morphology and dendritic growth	(+) possible to study individual neurons (+) useful for mechanistic studies (-) neurons are deprived of their normal afferent and efferent targets (-) maintenance of the cells is difficult (-) no blood-brain barrier	excitotoxic amino acids NMDA antagonists acrylamide lead acetate methylated halogens	Mayer and Westbrook, 1987 Choi, 1988 Hooisma, 1982 Holtzman *et al*, 1987 Davenport *et al*, 1989
Glial cell cultures	effects on myelinating cells and myelin sheaths; assessment of species-specific toxic effects on myelinating cells	(+) useful for mechanistic studies (-) no blood-brain barrier	ethanol alpha-chlorhydrin	Mithen *et al*, 1990 Evans and Fawthrop, 1990
Cell lines	effects on ion channels and interaction with receptors	(+) useful for studying cell biology (-) model system that shares only certain features with real neurons or glia	methylmercury lead organophosphates pyrethroid insecticides solvents acrylamide	Atchison and Schafer, 1990 Audesirk and Audesirk, 1990 Carrington *et al*, 1985 Oortgiesen *et al*, 1989 Selkoe *et al*, 1978 Walum *et al*, 1987
Organotypic explants	effects on development of the nerve system, on development of neuro-muscular junctions or other morphological endpoints	(+) useful for mechanistic studies (-) preparation and maintenance is difficult (-) neurons are immature (-) explant is disconnected from it's normal afferents	thallium, tellurium, triethyltin, methylmercury, chloroquine etc. hexacarbon solvents organophosphates	Yonezawa *et al*, 1980 Veronesi *et al*, 1984 Tuler and Bowen, 1989
Rotation-mediated aggregating cultures	effects on specific transmitter systems, on cell surface recognition and on enzymes	(+) ease of preparation, reproducibility and representation (+) appropriate for interdisciplinary investigation (-) neurons are immature (-) large quantities of foetuses are required (-) electrophysiological examination is not possible	ascorbic and kainic acid 6-hydroxydopamine, tricyclic antidepressants organophosphates methylmercury, cycloheximide, colchicine aluminium, ethylcholine mustard aziridinium	Trapp and Richelson, 1980 Majocha *et al*, 1981 Wehner *et al*, 1985 Jacobs *et al*, 1986 Atterwill and Collins, 1988 Atterwill, 1990

APPENDIX D SITE OF PRINCIPAL EFFECT OF SOME TOXIC CHEMICALS CAUSING MORPHOLOGIC CHANGES IN THE CENTRAL AND PERIPHERAL NERVOUS SYSTEM

Site of principal effect	Substance	Species effected	Reference
Central nervous system			
Vascular endothelium	lead	immature rat	Pentschew and Garro (1966)
	cadmium	immature rat, rabbit	Gabbiani *et al* (1967)
	tunicamycin	sheep	Berry and Vogel (1982)
	corynetoxins	immature rat	Finnie and O'Shea (1988)
	lovastatin	dog	Berry *et al* (1988)
Choroid plexus neuroepithelium	bis(4-amino-3-methylcyclohexyl) methane	rat	Ohshima *et al* (1984,1986)
	piperamide	rat	Levine (1977)
Ependymal neuroepethelium	amoscanate	rat	Krinke *et al* (1983)
Neurons	kainic acid	rat	Olney *et al* (1979)
	domoic acid	rat, monkey	Tryphonas and Iverson (1990)
	methyl mercury	rat, man	Chang (1977)
	acetyl ethyl tetramethyl tetralin (AETT)	rat	Spencer *et al* (1980c)
Astrocytes	6-aminonicotinimide	rat	Powell *et al* (1980)

Site of principal effect	Substance	Species effected	Reference
Oligodedroroglia and myelin sheaths	triethyl tin	rat, rabbit, man	Aleu *et al* (1963)
	hexachlorophene	rodents, sheep, dog, man	Towfighi and Gonatas (1976)
	2-3-dideocycytidine	rabbit	Anderson and Davidovich (1990)
	ethidium bromide	rat	Yazima and Suzuki (1979)
	cuprizone	rat, mouse	Suzuki and Kikkawa (1969)
	AETT	several spp. including man	Spencer *et al* (1980c)
Peripheral nervous system			
Vascular endothelium	lead	rat	Mizisin *et al* (1990)
Perineurium	2-chloroprocaine	rat	Myers *et al* (1986)
	ethanol, glycerol, ammonium sulphate	rat	Mizisin *et al* (1990)
Schwann cells and myelin sheaths	triethyl tin	rat, man, rabbit	Graham and Gonatas (1973)
	AETT	several sp. including man	Spencer *et al* (1980)
	tellurium	immature rat	Lampert and Garrett (1971)
	lead	rat, guinea pig	Fullerton (1966), Lampert and Schochet (1968)
	perhexilene maleate	man	Lhermite *et al* (1976)
	6-aminonictinamide	immature rat, rat	Brzoska and Adhami (1976)
	hexachlorophene	rodents, sheep, dog, man	Towfighi and Gonatas (1976)
Axons-proximal	iminodiproprionitrile	dog, rat	Griffin and Price (1980)

Site of principal effect	Substance	Species effected	Reference
Axons-distal	clioquinol	man, dog	Krinke *et al* (1979)
	acrylamide	man, baboon, cat, rat	cited by Spencer and Schaumburg (1976)
	alkyl, aryl phospahtes	rat, dog, hen, man	"
	diethyldi-thiocarbamate	hen, rabbit	"
	arsenic	man	"
	p-bromophenyl acetylurea	rat	"
	carbon disulphide	several laboratory species, man	"
	n-hexane, 2,5-hexanedione methyl n-butyl-ketone	man, rat	"
	isoniazid, other vitamin B6 inhibitors	man, chicken, dog, duck, rat	"
	nitrofurans	man, rat	"
	thalidomide	man	"
	thallium salts	man	"
	methyl mercury	rat	Cavanagh and Chen (1971)
Dorsal root ganglia	pyridoxine	dog, rat	Schaeppi and Krinke (1985), Krinke *et al* (1985)

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No. 2	The Mutagenic and Carcinogenic Potential of Formaldehyde
No. 3	Assessment of Test Methods for Photodegradation of Chemicals in the Environment
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